

# Differential role of nonhomologous end joining factors in the generation, DNA damage response, and myeloid differentiation of human induced pluripotent stem cells

Kerstin Felgentreff<sup>a</sup>, Likun Du<sup>b</sup>, Katja G. Weinacht<sup>a,c</sup>, Kerry Dobbs<sup>a</sup>, Margarita Bartish<sup>b</sup>, Silvia Giliani<sup>d</sup>, Thorsten Schlaeger<sup>c</sup>, Alexander DeVine<sup>c</sup>, Axel Schambach<sup>c,e</sup>, Lisa J. Woodbine<sup>f</sup>, Graham Davies<sup>g</sup>, Sachin N. Baxi<sup>a</sup>, Mirjam van der Burg<sup>h</sup>, Jack Bleesing<sup>i</sup>, Andrew Gennery<sup>j</sup>, John Manis<sup>k</sup>, Qiang Pan-Hammarström<sup>b</sup>, and Luigi D. Notarangelo<sup>a,l,1</sup>

<sup>a</sup>Division of Immunology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115; <sup>b</sup>Division of Clinical Immunology and Transfusion Medicine, Department of Laboratory Medicine, Karolinska Institutet at Karolinska University Hospital Huddinge, SE 14186 Stockholm, Sweden; <sup>c</sup>Division of Pediatric Hematology/Oncology, Boston Children's Hospital and Dana Farber Cancer Institute, Boston, MA 02115; <sup>d</sup>A. Nocielli Institute for Molecular Medicine, Pediatric Clinic, University of Brescia, Spedali Civili, 25123 Brescia, Italy; <sup>e</sup>Institute of Experimental Hematology, Hannover Medical School, D-30625 Hannover, Germany; <sup>f</sup>Genome Damage and Stability Centre, University of Sussex, Brighton BN1 9RQ, United Kingdom; <sup>g</sup>Centre for Immunodeficiency, Great Ormond Street Hospital and Institute of Child Health, London WC1N 3JH, United Kingdom; <sup>h</sup>Department of Immunology, Erasmus Medical Center, 3015 GE, Rotterdam, The Netherlands; <sup>i</sup>Division of Bone Marrow Transplant and Immunodeficiency, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229; <sup>j</sup>Institute of Cellular Medicine, Pediatric Immunology, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne NE1 4LP, United Kingdom; <sup>k</sup>Department of Transfusion Medicine, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115; and <sup>l</sup>Harvard Stem Cell Institute, Cambridge, MA 02138

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**Nonhomologous end-joining (NHEJ) is a key pathway for efficient repair of DNA double-strand breaks (DSBs) and V(D)J recombination. NHEJ defects in humans cause immunodeficiency and increased cellular sensitivity to ionizing irradiation (IR) and are variably associated with growth retardation, microcephaly, and neurodevelopmental delay. Repair of DNA DSBs is important for reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). To compare the specific contribution of DNA ligase 4 (LIG4), Artemis, and DNA-protein kinase catalytic subunit (PKcs) in this process and to gain insights into phenotypic variability associated with these disorders, we reprogrammed patient-derived fibroblast cell lines with NHEJ defects. Deficiencies of LIG4 and of DNA-PK catalytic activity, but not Artemis deficiency, were associated with markedly reduced reprogramming efficiency, which could be partially rescued by genetic complementation. Moreover, we identified increased genomic instability in LIG4-deficient iPSCs. Cell cycle synchronization revealed a severe defect of DNA repair and a G0/G1 cell cycle arrest, particularly in LIG4- and DNA-PK catalytically deficient iPSCs. Impaired myeloid differentiation was observed in LIG4-, but not Artemis- or DNA-PK-mutated iPSCs. These results indicate a critical importance of the NHEJ pathway for somatic cell reprogramming, with a major role for LIG4 and DNA-PKcs and a minor, if any, for Artemis.**

**D**NA repair is a crucial process for cell integrity, and its failure may result in cell cycle arrest, apoptosis, senescence, and introduction of genomic abnormalities that may lead to neoplastic transformation (1). Cellular DNA damage occurs frequently and can be caused by exogenous factors, such as exposure to ionizing and UV radiation and chemical drugs, or may result from endogenous sources, in particular reactive oxygen species (ROS) and replication errors (2). Although these insults may lead to both DNA single-strand breaks (SSBs) and double-strand breaks (DSBs), the latter are more critical in terms of cell survival and mutation probability. Importantly, DNA DSBs are also physiologically introduced in the T-cell receptor (TCR) and Ig genes during V(D)J recombination and class switch recombination (3).

Homologous recombination (HR) and nonhomologous end joining (NHEJ) represent two major pathways of DNA DSB repair in mammalian cells (4). HR is a high fidelity mechanism that requires a sister chromatid as a template and therefore is restricted to late S and G2 phases of the cell cycle. NHEJ is the major repair pathway in mammalian somatic cells, operating in G0/G1 phases of the cell cycle and competing with HR in the late

S and G2 phases (5). The Ku70/Ku80 heterodimer binds rapidly at DNA DSBs, resulting in recruitment of two DNA-dependent protein kinase catalytic subunit (DNA-PKcs) molecules and formation of the DNA-PK holoenzyme. In the classical NHEJ (C-NHEJ) pathway, DNA-PKcs activates the endonuclease Artemis, which processes the DNA ends with overhangs. Finally, the XRCC4-DNA ligase 4 (LIG4) complex is recruited and ligates the DNA strand with the help of the XRCC4-like factor (XLF) (2). In addition to XRCC4-LIG4-dependent C-NHEJ, at least one alternative end-joining (A-EJ) pathway exists, which involves microhomology (MH) and is mainly used in cells with defects affecting C-NHEJ (6).

Consistent with the key role played by NHEJ in V(D)J recombination, severe defects of NHEJ in humans result in severe combined immunodeficiency (SCID) with lack of T and B lymphocytes, as well as increased radiation sensitivity and a variable spectrum of extraimmune manifestations (7).

The majority of patients with radiation-sensitive SCID carry biallelic mutations in the DNA Cross-Link Repair 1C (*DCLRE1C*)

## Significance

**In this study, we compare the differential impact of non-homologous end-joining-deficient induced pluripotent stem cells (iPSCs) derived from patients with mutations in genes encoding DNA ligase 4, DNA-protein kinase catalytic subunit, or Artemis, on cellular reprogramming, DNA damage repair, and myeloid differentiation. We found a predominant role for ligase 4 in genomic stability and myeloid differentiation of iPSCs, but a minor one for Artemis. Disease modeling with patient-derived iPSCs may provide significant insights into phenotypic variability associated with mutations in various genes along the same pathway and may serve as a platform to explore tissue-specific consequences of single gene defects and development of therapeutic interventions.**

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<sup>1</sup>To whom correspondence should be addressed. E-mail: Luigi.Notarangelo@childrens.harvard.edu.

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gene encoding for Artemis. This condition is associated with mild cellular radiosensitivity but no evidence of bone marrow failure or symptoms of neurodevelopmental delay. By contrast, LIG4 deficiency results in a more complex syndrome with growth retardation, pancytopenia, microcephaly, developmental delay, and a tendency to develop bone marrow failure and malignancies (8). Patients with LIG4 deficiency carry a hypomorphic mutation on at least one of the two mutated alleles. Homozygous deletion of the *Lig4* gene in mice is embryonically lethal due to elevated apoptosis in neuronal stem cells and progenitor cells (9). Deficiency of XLF causes combined immunodeficiency, associated with microcephaly and developmental delay (10). Finally, only two patients with mutations of the Protein Kinase, DNA-activated, Catalytic polypeptide (*PRKDC*) gene, encoding for DNA-PKcs, have been identified so far. The first patient carried a homozygous mutation that affected activation of Artemis protein, but left DNA-PK catalytic activity intact; this patient presented with an isolated T<sup>-</sup>B<sup>-</sup>NK<sup>+</sup> SCID phenotype (11). More recently, a second patient has been reported, whose *PRKDC* mutations dramatically reduced DNA-PK catalytic activity. This patient presented with severe and progressive neurodevelopmental delay, microcephaly, and dysmorphisms, in addition to T<sup>-</sup>B<sup>-</sup>NK<sup>+</sup> SCID (12). Overall, these data indicate that the clinical phenotype associated with defects of NHEJ in humans may vary, depending on the nature of the gene affected and the specific mutations.

Induced pluripotent stem cells (iPSCs) represent a novel and powerful platform for disease modeling. Somatic cells can be reprogrammed into iPSCs by overexpression of the four factors OCT4, SOX2, KLF4, and c-MYC (13). However, reprogramming imposes cellular stress by induction of significant changes of gene expression (14) and enhanced cell metabolism, leading to increased levels of ROS (15). Reprogramming-associated DNA DSBs trigger the DNA damage response (DDR) and up-regulate the p53 pathway, thereby promoting cell death and senescence and limiting the efficiency of cell reprogramming (16, 17). Consistent with this, impaired reprogramming efficiency has been reported for human Fanconi anemia (FA) cells (18), LIG4- and XLF-mutated human fibroblasts (19, 20), and Ataxia Telangiectasia Mutated (ATM)-deficient mouse embryonic fibroblasts (MEFs) (21).

In this study, we used fibroblasts and iPSCs from patients with different mutations in various NHEJ genes (*DCLRE1C*, *LIG4*, and *PRKDC*) to model heterogeneity of the disease phenotype and to compare the impact of naturally occurring mutations in these genes on somatic cell reprogramming, NHEJ function, DDR, and myeloid differentiation in vitro.

## Results

**Defects of LIG4- and DNA-PK Catalytic Activation, but Not Artemis, Severely Reduce Efficiency of Reprogramming.** To investigate the impact of NHEJ on the efficiency and quality of reprogramming, we took advantage of a series of fibroblast cell lines derived from

patients carrying mutations in *DCLRE1C* (Artemis), *PRKDC* (DNA-PKcs), or *LIG4* genes and presenting with a variable severity of the clinical phenotype (Table 1). For each of these lines, a typical profile of DDR was observed on exposure to ionizing irradiation (IR) and up to 48 h after irradiation (Fig. S1).

Fibroblast lines were transduced with a lentiviral vector containing the 4-in-1 transcription factor cassette (OKSM) and dTomato as a reporter (22). After transduction at a multiplicity of infection (MOI) of 1–2, we were able to obtain iPSC colonies from each fibroblast line. All iPSC lines tested expressed pluripotency markers as shown by immunofluorescence staining (Fig. S24) and quantitative PCR (qPCR) (Fig. S24) and were confirmed to carry the same mutations as in parental fibroblasts (Fig. S3). Artemis-deficient fibroblasts and the fibroblast cell line ID177, whose *PRKDC* mutation affects Artemis activation, showed reprogramming efficiencies similar to control iPSCs (4.75%) (Fig. 14). By contrast, only one morphologically intact iPSC clone could be recovered after a prolonged period from each of the LIG4-deficient lines 411BR, F07614, and SCID072. Eight iPSC colonies were obtained from the LIG4-deficient line HYGM022, which was derived from a patient with a less severe phenotype (Table 1). The DNA-PK catalytically defective line NM720 was also reprogrammed with markedly reduced efficiency (0.1%), albeit higher than what observed for LIG4-deficient lines (Fig. 14).

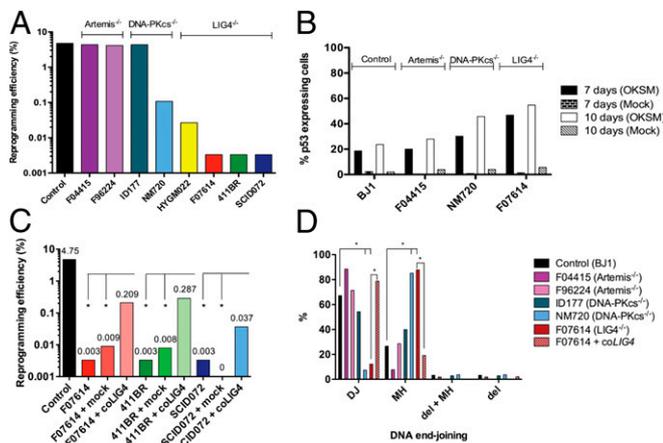
Because activation of the p53 pathway by DSBs is known to account for impaired survival of LIG4-deficient mice (9) and constrains efficiency of somatic cell reprogramming (16), we analyzed whether the reduced reprogramming efficiency of LIG4-mutated and of DNA-PK catalytically defective cells was due to increased and prolonged induction of p53. To test this, Artemis-, DNA-PKcs-, LIG4-deficient, and control fibroblasts were transduced with the OKSM-containing reprogramming vector and, in parallel, with a mock vector lacking the 4-in-1 transcription factor cassette. Cells were sorted for dTomato expression 48 h after transduction and analyzed for p53 expression by immunofluorescence staining after 7 and 10 d of culture. Marked up-regulation of p53 expression was observed after 10 d of culture in DNA-PKcs- (NM720) and LIG4-mutated (F07614) lines, (45% and 55%, respectively) compared with control fibroblasts (23%) and Artemis-deficient cells (28%) (Fig. 2B). By contrast, no significant induction of p53 was observed on transduction with the mock vector in any of the lines tested. These data rule out that the abnormal response of F07614 and NM720 lines could reflect DNA damage following lentivirus integration and rather indicate a critical role for epigenetic modifications and cell stress in inducing p53 expression and constraining efficiency of somatic cell reprogramming (23).

**LIG4-Deficient Cells Tend to Acquire Clonal Karyotypic Abnormalities During Culture and/or Reprogramming.** Karyotype analysis was performed on two to eight clones of each iPSC line, as well as on the parental fibroblasts. Two of four LIG4-deficient iPSC lines showed clonal karyotypic abnormalities; in some, chimerism was

**Table 1. Cell lines and patient characteristics**

Patient code	Gene	Mutation (allele 1; 2)	Immunological phenotype	Extraintestinal manifestations	Reference
F04415	<i>DCLRE1C</i>	del exon 1–3; del exon 1–3	T <sup>-</sup> B <sup>-</sup> NK <sup>+</sup> SCID	None	
F96224	<i>DCLRE1C</i>	G126D; L70del	T <sup>lo</sup> B <sup>lo</sup> CID	None	(42)
ID177	<i>PRKDC</i>	L3062R; L3062R	T <sup>-</sup> B <sup>-</sup> NK <sup>+</sup> SCID	None	(12)
NM720	<i>PRKDC</i>	A3574V; del exon 16	T <sup>-</sup> B <sup>-</sup> NK <sup>+</sup> SCID	Severe microcephaly, sensorineural deafness, visual impairment	(13)
HYGM022	<i>LIG4</i>	R814X; K449Q	B <sup>lo</sup>	Microcephaly	
F07614	<i>LIG4</i>	A3V, T9I, R278H; A3V, T9I, R278H	T <sup>lo</sup> B <sup>-</sup> CID, hyper-IgM	Microcephaly, mild developmental delay	(26)
411BR	<i>LIG4</i>	A3V, T9I, R278H; A3V, T9I, R278H	T <sup>-</sup> B <sup>lo</sup> NK <sup>+</sup> CID	Microcephaly, developmental delay, non-EBV lymphoma	(8, 9)
SCID072	<i>LIG4</i>	Q558P; K424fs	T <sup>lo</sup> B <sup>lo</sup> CID, pancytopenia	Microcephaly, IUGR, developmental delay	

IUGR, intrauterine growth retardation.



**Fig. 1.** Reprogramming efficiency of NHEJ-deficient fibroblasts. (A) Human Artemis-, DNA-PKcs-, and LIG4-deficient fibroblasts were reprogrammed to iPSCs by lentiviral-mediated vector transduction. Reprogramming efficiency was calculated as the percentage of morphologically intact iPSC colonies obtained per total number of fibroblasts transduced. (B) Fibroblasts from given cell lines were either OKSM- or mock-transduced and sorted for dTomato expression after 48 h. Percentage of p53-expressing cells was assessed by immunofluorescence after 7 and 10 d of culture. (C) LIG4-deficient fibroblasts transduced with either human codon optimized LIG4 cDNA (*coLIG4*)-expressing vectors or with mock vectors were reprogrammed to iPSCs. Complementation of LIG4 partially rescued the reprogramming efficiency obtained in the previous experiments ( $*P \leq 0.05$ ). (D) The percentages of plasmids repaired by direct joining (DJ), microhomology-mediated joining (MH), combinations of MH and deletions (MH + del), or deletions (del) not associated with MH or insertion were analyzed in iPSCs transfected with the linearized pDVG94 construct and are reported for given cell lines ( $*P \leq 0.05$ ).

documented in parental fibroblasts, whereas others were apparently acquired de novo during reprogramming and/or expansion of iPSCs (Table S1). By contrast, no karyotypic abnormalities were identified in any of the other iPSC lines tested. These findings of increased genomic instability in LIG4 deficiency are consistent with a recent report (21).

**Lentivirus-Mediated Complementation of LIG4-Deficient Fibroblasts Restores Cellular Radiosensitivity and NHEJ Activity and Partially Rescues Reprogramming Efficiency.** LIG4-deficient fibroblast lines were transduced with a lentiviral vector expressing human codon-optimized *LIG4* (*coLIG4*) cDNA, along with cDNAs of *EGFP* and blasticidin resistance (*BSD*), or with a lentiviral vector expressing *EGFP* and *BSD* only (mock). GFP-expressing fibroblasts were sorted and grown in blasticidin-containing medium. Both *coLIG4*- and mock-transduced fibroblasts were then tested for  $\gamma$ H2AX nuclear foci formation in response to IR with 5 Gy to confirm complementation of LIG4 deficiency, which rescued the radiation sensitivity phenotype (Fig. S4). Subsequently, *coLIG4*- and mock-complemented 411BR, F07614, SCID072, and HYGM022 fibroblast cell lines were reprogrammed for iPSC generation. Although reprogramming efficiency of mock-transduced lines remained very low, partial rescue of reprogramming efficiency was observed for the *coLIG4*-complemented lines (Fig. 1C).

Real-time PCR confirmed that the *coLIG4*-complemented iPSC lines expressed pluripotency markers, as shown in Fig. S5 for iPSCs obtained from the F07614 line. However, karyotypic analysis revealed chromosomal abnormalities (Table S1). Some of these were acquired during the process of fibroblast complementation or mock transduction and others during reprogramming.

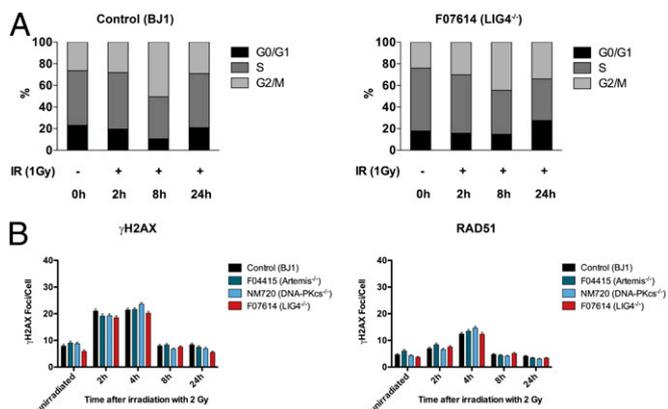
**LIG4 and DNA-PKcs, but Not Artemis, Are Critical for NHEJ Activity in Pluripotent Stem Cells.** We tested iPSC lines for their ability and efficiency to perform NHEJ using a plasmid ligation assay (24).

A linearized plasmid with blunt ends harboring a 6-bp sequence homology was transfected into cells, and plasmid DNA was recovered after 24 h. When repair junctions from joined plasmids were sequenced, limited use of direct joining was observed in LIG4-deficient and in the DNA-PK catalytically defective iPSCs, which mainly used MH-mediated joining. This pattern was reversed toward WT in *coLIG4*-complemented F07614 iPSCs (Fig. 1D). By contrast, the pattern of end-joining was similar in control iPSCs and in both the Artemis-deficient lines F04415 and F96224, as well as in the DNA-PK-mutated line ID177, in which Artemis activation is affected, but DNA-PK catalytic activity is intact (Fig. 1D).

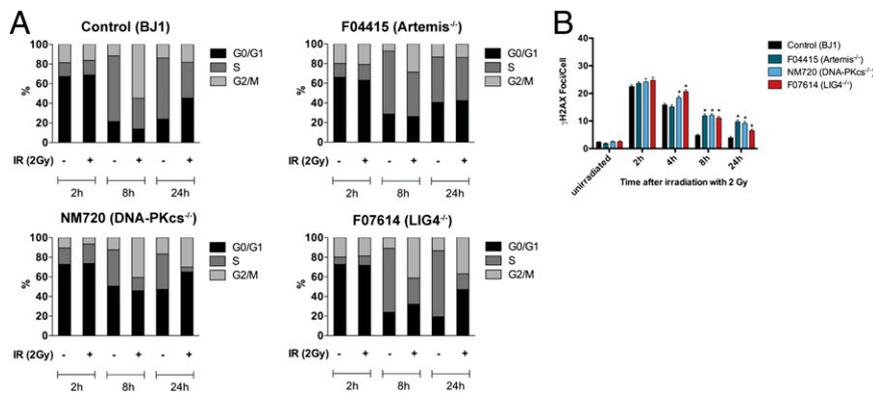
**Nonsynchronized LIG4-Deficient iPSCs Do Not Show Enhanced Abnormalities of DDR on Exposure to Ionizing Irradiation.** It has been reported that embryonic stem (ES) cells and iPSCs are highly susceptible to IR with increased apoptosis and cell cycle arrest in G2/M (25, 26). Consistent with this, when we exposed control and patient-derived iPSCs to IR, even at low dosage (0.5–1 Gy), we observed massive apoptosis and severe disruption of colonies (Fig. S6).

To test DDR in NHEJ-deficient iPSCs, initially we evaluated cell cycle progression in control and LIG4-deficient F07614 nonsynchronized, unirradiated cells and at 2, 8, and 24 h after irradiation (1 Gy). No significant differences were detected in the cell cycle distribution of control vs. LIG4-deficient iPSCs at any time point before or after IR (Fig. 2A and Fig. S7). We hypothesized that the DNA DSBs repair in nonsynchronized control and NHEJ-deficient iPSC lines occurred predominantly by HR rather than NHEJ. To test for this, we analyzed RAD51 together with  $\gamma$ H2AX nuclear foci formation. The number of  $\gamma$ H2AX foci increased early (30 min) after IR and declined to baseline levels after 8 h. As previously described (27), RAD51 foci formation peaked at 2 h and returned to baseline levels by 8 h after IR (Fig. 2B). Around 60–80% of iPSCs showed colocalization of RAD51 and  $\gamma$ H2AX nuclear foci at all of the time points, suggesting a frequent use of HR for DNA DSB repair in iPSCs. Furthermore, we observed frequent colocalization of  $\gamma$ H2AX and RAD51 foci also in unirradiated iPSCs, consistent with their high replicative activity.

**On Cell Cycle Synchronization and Exposure to IR and LIG4 and DNA-PK Catalytically Deficient iPSCs, Arrest in G0/G1 and NHEJ-Deficient iPSCs Reveal a DNA Repair Defect.** Because the high replicative activity of iPSCs could confound interpretation of  $\gamma$ H2AX foci



**Fig. 2.** Cell cycle and DNA damage response in nonsynchronized iPSCs. (A) The percentages of cells in G0/G1, S, and G2/M phases of the cell cycle are shown for both unirradiated cells and for cells exposed to 1-Gy IR at 2, 8, and 24 h of culture. (B) Nonsynchronized iPSCs from indicated cell lines were irradiated (0.5 Gy) and fixed at given time points. Nuclear  $\gamma$ H2AX and RAD51 foci formation were analyzed by coimmunostaining and confocal microscopy. Results were obtained in multiple sets of experiments and are expressed as mean numbers of foci per cell  $\pm$  SEM.



**Fig. 3.** Cell cycle and DNA damage response in synchronized iPSCs. (A) Indicated cell lines were synchronized and irradiated with 2 Gy, and cell cycle was analyzed by flow cytometry. Diagrams display the percentages of live cells in G0/G1, S, and G2/M cell cycle phases at 2, 8, and 24 h under nonirradiated and irradiated (2 Gy) conditions. Data shown are representative for results from three independent experiments. (B) Mean numbers of  $\gamma$ H2AX foci per cell + SEM are shown for indicated iPSC lines at all time points tested ( $*P \leq 0.05$ ).

formation data, we synchronized cell cycles with nocodazole for 16 h (28). Two hours after release from synchronization, about 70% of iPSCs were in G0/G1 phase. In the absence of irradiation, control and NHEJ-deficient iPSCs resumed high proliferative activity. On irradiation with 2 Gy, significant differences in cell cycle progression were observed among iPSC lines. In particular, compared with control and Artemis-deficient lines, the NM720 and F07614 iPSC lines showed fewer cells in S phase at 8 and 24 h, and a large fraction (45–60%) of cells were in G0/G1 at 24 h after exposure to IR (Fig. 3A).

Next, we analyzed  $\gamma$ H2AX foci at 2, 4, 8, and 24 h after 2-Gy irradiation of control and NHEJ-deficient synchronized iPSCs. To distinguish between cells in G0/G1 and G2/M, cells were stained for the centromere protein CENP-F (29). Compared with control irradiated lines, the mean numbers of  $\gamma$ H2AX foci per cell declined with slower kinetics in all NHEJ-deficient iPSC lines that were in G0/G1 (Fig. 3B and Fig. S8).

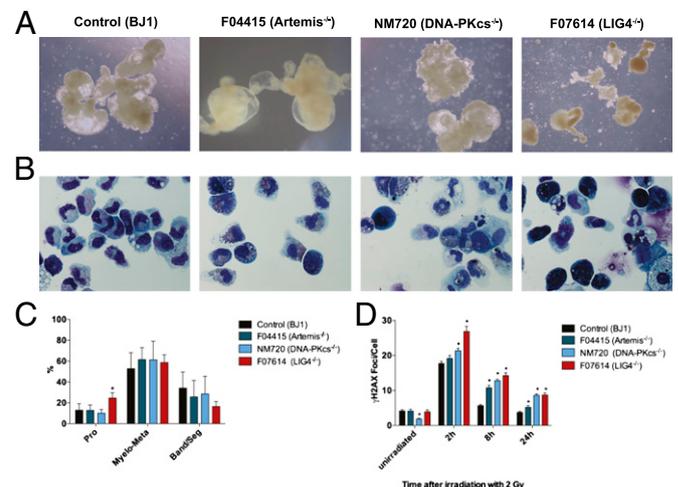
**LIG4-Deficient iPSCs Have Reduced Potential for Hematopoietic Differentiation Toward the Myeloid Lineage.** LIG4 deficiency is associated with an increased risk of bone marrow failure (8). Progressive loss of hematopoietic stem and progenitor cells has been demonstrated in a mouse model of the disease (30). We tested the ability of F07614 LIG4-mutated iPSCs to expand and generate embryoid bodies (EBs). In multiple experiments, we observed that F07614 LIG4-mutated iPSCs yielded poorly developed EBs and embryoid sacs, with features of apoptosis (Fig. 4A). To test and compare the potential of NHEJ-deficient iPSCs for hematopoietic differentiation, we performed a colony forming unit (CFU) assay. The total numbers of CFUs obtained from F07614 EBs were reduced to 52–54% compared with control iPSCs, and morphological analysis of granulocyte maturation revealed skewing to more immature stages, with higher frequency of promyelocytes in LIG4-deficient compared with control iPSCs (Fig. 4B and C). By contrast, the efficiency of myeloid differentiation was not altered in Artemis (F04415)- and DNA-PKcs (NM720)-deficient cell lines. However, myeloid cells derived from all NHEJ-deficient iPSCs showed increased and persistent formation of  $\gamma$ H2AX foci on irradiation with 2 Gy (Fig. 4D), recapitulating what was observed in parental fibroblasts and synchronized iPSCs.

## Discussion

Cellular reprogramming is associated with DNA and histone modifications and massive induction of gene expression. Although the integration of viral vectors delivering reprogramming transgenes requires DNA DSBs repair (31), it has been demonstrated that DNA DSBs are also generated when nonintegrating systems are used for somatic cell reprogramming (23). This

likely reflects significant cellular and genotoxic stress, which leads to up-regulation of the p53 pathway (16) and induction of cell death and senescence (32) and thus somatic cell reprogramming efficiency.

By comparing side-by-side human cell lines with different mutations in a broader panel of NHEJ genes, our data demonstrate for the first time important differences in the requirement for specific components of NHEJ and of specific mutations in these genes during somatic cell reprogramming, DDR, and in vitro differentiation of iPSCs. In particular, we observed increased expression of p53 and severe impairment of somatic cell reprogramming on transduction of LIG4- and DNA-PK catalytically deficient fibroblasts with the lentiviral reprogramming vector. By contrast, normal reprogramming efficiency and levels of p53 expression comparable to control were observed for Artemis-deficient fibroblasts and for the line ID177, whose *PRKDC* homozygous mutation affects Artemis activation, but leaves DNA-



**Fig. 4.** Differentiation efficiency and DNA damage response of myeloid cells derived from NHEJ-deficient iPSCs. (A) EBs were developed from human control, Artemis-, catalytically DNA-PK- and LIG4-deficient iPSCs. One representative experiment of five is shown. (B) EBs from the indicated cell lines were cultured in methylcellulose and granulocyte colonies (CFU-G) were picked after 14 d. (C) Granulocyte maturation was assessed based on nuclear and cytoplasmic morphology and divided into groups of promyelocytes, myelocytes/metamyelocytes, and bands/segmented cells ( $*P \leq 0.05$ ). (D) Myeloid cells (CFU-G, CFU-M, and CFU-GM) of indicated cell lines were irradiated with 2 Gy, and  $\gamma$ H2AX foci formation is expressed as mean foci numbers per cell + SEM ( $*P \leq 0.05$ ).

PK catalytic activity intact (12). Altogether, these data demonstrate a critical role for the NHEJ machinery for DNA repair and survival in cells undergoing reprogramming.

It has been reported that the spectrum of chromosomal instability is different and less severe in Artemis-deficient cells compared with LIG4- or DNA-PKcs-deficient lymphocytes (33). Artemis plays a role only in a subset of NHEJ events, in particular processing DNA ends with overhangs or opening the hairpin (34), and it is involved in an ATM-dependent NHEJ pathway repairing with slower kinetics (35). Our data extend these observations by demonstrating that Artemis is dispensable for somatic cell reprogramming and genomic stability, although its role in NHEJ can be appreciated in synchronized iPSCs at later time points (8–24 h) after IR.

In the absence of C-NHEJ, DNA DSBs may be joined by the A-EJ pathway (6). This process facilitates chromosomal translocations with MH joins at the junctions (36). We observed increased genomic instability, including emergence of structural chromosomal abnormalities, in iPSC lines derived from LIG4-deficient fibroblasts, and similar results have been recently reported by others (19). In particular, several chromosomal abnormalities have been identified in LIG4-mutated iPSCs that were not present in parental fibroblasts. These chromosomal abnormalities may have occurred at various stages during iPSC generation and culture, including fibroblast transduction, reprogramming, and/or iPSC expansion. We used fluorescent in situ hybridization to analyze the parental fibroblast lines for any translocations that had been observed in the derived iPSCs (Table S1). However, because of the limited number of cells analyzed, chromosomal abnormalities possibly present at very low frequency in the parental fibroblasts may have been missed. Although genomic instability and mutagenesis have been reported in *Prkdc*<sup>-/-</sup> mouse models (37, 38), no chromosomal abnormalities were detected in iPSCs deficient in DNA-PK catalytic activity. Lack of chromosomal abnormalities in DNA-PK catalytically deficient fibroblasts and iPSCs studied here may be due to the low level of spontaneous genomic instability or to tissue specificity, as suggested by the observation that *p53*<sup>-/-</sup> *scid* mice predominantly develop lymphoma from erroneously repaired programmed DNA breaks (39).

Using a plasmid ligation assay, we were able to confirm defective C-NHEJ activity and preferential use of the A-EJ pathway with a higher proportion of MH joins in LIG4-deficient iPSCs, thus recapitulating a hallmark of this disease (6). Increased proportion of MH joins was observed also in NM720 DNA-PK catalytically deficient iPSCs. Large deletions are also expected to occur during joining of DNA DSBs in DNA-PKcs-deficient cells; however, the plasmid ligation assay does not permit to properly identify large deletions and score their relative frequency.

Pluripotent stem cells are characterized by high proliferative activity with a large proportion of cells in S phase of the cell cycle and shortened G1 and G2 phases, which is important for maintaining pluripotency (40) and favors use of HR for DNA DSB repair (27). Consistent with these observations, we found around 50% of unirradiated nonsynchronized control- and NHEJ-deficient iPSCs in S phase and observed no significant difference among nonsynchronized NHEJ-deficient and control iPSCs in both cell cycle and DDR on irradiation. However, it has been shown on synchronized human ES cells (ESCs) irradiated with UV C (UVC) (28) and on human nonsynchronized iPSCs in response to IR (27) that pluripotent stem cells are capable to interrupt cell cycle progression in response to DNA damage. To investigate more properly the DDR of NHEJ-defective cells, we performed cell cycle synchronization, and exposed iPSCs to IR. Under these conditions, a more severe phenotype (cell cycle arrest at the G0/G1 checkpoint) was observed in iPSCs with LIG4 deficiency or impaired DNA-PK catalytic activity but not in Artemis-deficient iPSCs.

Defective EB generation and impaired myeloid differentiation was observed for F07614 LIG4-mutated iPSCs. Along with

similar data generated in mice (30), these observations indicate that impaired proliferation of hematopoietic stem and progenitor cells is responsible for the increased risk of bone marrow failure in LIG4 deficiency. The decreased numbers of in vitro-differentiated myeloid cells we observed is likely due to the loss of progenitor cells as evidenced by the smaller and fewer EBs generated. By contrast, no defects of EB generation and myeloid differentiation were observed in Artemis- and DNA-PK catalytically deficient iPSCs. The relative increase in the percentage of promyelocytes among differentiated myeloid cells may be due to the proliferative defect we see in EB development, with an attrition of more mature myeloid forms.

In summary, we demonstrated that NHEJ is critical for somatic cell reprogramming and maintenance of genomic stability in iPSCs and that use of HR may obscure defects of DNA DSB repair in nonsynchronized NHEJ-deficient iPSCs. Importantly, we showed that important differences exist in the requirement for individual NHEJ factors in somatic cell reprogramming, as well as in iPSC differentiation and DDR. These gene- and mutation-specific differences of the biological behavior of NHEJ-defective iPSCs model distinctive features of the clinical phenotype observed in patients with these disorders, including bone marrow failure and susceptibility to malignancies. Differentiation of NHEJ-deficient iPSC lines to other lineages, such as neurons, may help model additional phenotypic differences observed in vivo in patients.

## Materials and Methods

**Cell Lines and Cell Culture.** Fibroblast cell lines were grown from punch skin biopsies, collected on informed consent under protocol 0409113R approved by Children's Hospital Institutional Review Board (IRB) or under IRB-approved protocols at other collaborating institutions and made available to L.D.N. under protocol 509-08-0435. Culture conditions for fibroblasts and iPSCs have been previously described (41).

**Vector Cloning, Lentiviral Vector Production, and Gene Complementation.** Human fibroblasts were reprogrammed by transduction with a self-inactivating lentiviral vector containing the four transcription factors *OCT4*, *SOX2*, *KLF4*, and *c-MYC* (OKSM) and dTomato cDNA (22). Cloning of the complementation vector for LIG4 deficiency, lentiviral vector production, and genetic complementation of LIG4-deficient fibroblasts are described in *SI Materials and Methods*.

**Reprogramming, iPSC Generation, and Assessment of Reprogramming Efficiency.** Viral transduction was performed in  $3 \times 10^4$  fibroblasts at MOIs of 1 for the original fibroblast lines and 2 for the complemented lines as described. Reprogramming efficiencies were calculated by dividing the number of morphologically intact iPSC colonies obtained after 21 d by the number of fibroblasts transduced.

**Plasmid Ligation NHEJ Assay.** The NHEJ assay was performed as previously described (24). The linearized plasmid pDVG94 with defined, blunt DNA ends with 6-bp direct repeats (ATCAGC) was transiently transfected into single cell suspensions of iPSCs by nucleofection (Amaxa Nucleofector and Cell Line Nucleofector Kit V; Lonza). Plasmid DNA was recovered after 24 h, and the recombination junctions were PCR amplified using primers FM30 and DAR5 (24). The resulting PCR products were gel purified, cloned into pGEM-T vectors, and sequenced by Sanger sequencing.

**Synchronization of iPSCs and Cell Cycle Analysis.** iPSCs grown under feeder-free conditions were synchronized by treatment with 200 ng/mL nocodazole in culture media for 16 h, after which medium was replaced by mTESR without nocodazole, and cells were immediately irradiated with 2 Gy. For cell cycle analysis (Apoptosis, Cell Damage and Proliferation Kit; BD Biosciences), cells were pulsed with 10  $\mu$ M BrdU for 45 min at 37 °C, enzymatically detached with Accutase (STEMCELL), and analyzed by flow cytometry at indicated time points. Nonirradiated cells fixed at same time points served as controls.

**Irradiation, Immunocytochemistry, and Confocal Microscopy.** Irradiated fibroblasts (5 Gy) and iPSCs (0.5–2 Gy) were stained for anti-phospho Ser-139 Histone H2AX and RAD51 or CENP-F, respectively, and subsequently with Alexa fluor anti-mouse/anti-rabbit 488/594 antibodies, as described in *SI Materials and Methods*. Confocal imaging was performed on a Fluoview FV 1000 microscope (UplanApo 10 $\times$  and 60 $\times$ /1.2-NA water lenses; Olympus).

The numbers of nuclear foci per cell were counted manually or with Volocity software (PerkinElmer) after careful setup.

To assess p53 expression in reprogramming fibroblasts, OKSM- and mock-transduced cells were sorted for dTomato expression and stained with anti-p53 (mouse monoclonal, clone DO-1, sc-126; Santa Cruz) and Alexa fluor anti-mouse 647 (Invitrogen) antibodies after 7 and 10 d of culture. Imaging was performed on a BD Pathway 435 bioimager (20 $\times$  magnification).

**Hematopoietic Differentiation of iPSCs Toward the Myeloid Lineage.** Development of EBs and myeloid differentiation were performed as described in *SI Materials and Methods*. For morphological analysis of granulocyte maturation, CFU-G colonies were selectively harvested, and cells were spun on slides and stained with May-Grunwald Giemsa. Random fields were acquired and analyzed on an Eclipse E800 microscope (Nikon) with bright field illumination (20 $\times$ ), and at least 1,000 cells were counted per cell line and experiment. DNA repair efficiency was analyzed on myeloid cells irradiated with 2 Gy in methylcellulose cultures and harvested on slides at the indicated time points. Assessment of  $\gamma$ H2AX foci formation was performed as described above.

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