Supporting Information

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SI Materials and Methods

Vector Cloning and Lentiviral Vector production. To generate a complementation vector for ligase 4 (LIG4) deficiency, the human *LIG4* sequence was codon optimized (co*LIG4*) and engineered with a Kozak sequence (GeneArt; Invitrogen). After PCR amplification (primer sequences listed below), the cDNA was cloned into a lentiviral vector backbone containing the spleen focus-forming virus U3 promoter and an internal ribosome entry site-EGFP-Bsd cassette for selection, provided by A. Schambach (Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany). For cloning, the inFusionHD EcoDry kit (Clontech) was used according to the manufacturer's instructions. Additionally, a mock vector expressing EGFP-Bsd was created from the same backbone. Lentiviral vector production was performed in 293T packaging cells (1). Fluorochrome expression was measured 48–72 h after transduction into BJ1 fibroblasts by flow cytometry on an *LSR*-Fortessa (BD Bioscience) and virus titers were calculated as described (2).

Genetic Complementation of LIG4-Deficient Fibroblasts. A total of 10^5 fibroblasts were transduced with either co*LIG4* or mock vectors at a multiplicity of infection (MOIs) of 2 in human fibroblast medium supplemented with 5 μg/mL protamine sulfate (Sigma-Aldrich), 50 μg/mL vitamin C (Sigma), and 2 mM valproic acid (Sigma). Three days after transduction, complemented fibroblasts were expanded under antibiotic selection with 8 μg/mL blasticidin (Invitrogen). GFP-expressing cells were sorted on a 3 Laser FACSAria IIU (BD Bioscience) using an 85-μm nozzle at 40 PSI and were subsequently used for induced pluripotent stem cell (iPSC) generation, radiosensitivity testing, and karyotype analysis.

Immunohistochemistry of Pluripotency Markers in iPSCs. Morphologically intact iPSC colonies were subcloned and characterized for the expression of pluripotent markers by immunofluorescence staining for TRA-1-60, SSEA-4, NANOG, OCT4, and Hoechst (3). Imaging was performed on a BD Pathway 435 bioimager with 10× magnification (BD Bioscience).

Quantitative Real-Time PCR. RNA was extracted from generated iPSCs using the mirVana RNA isolation kit (Ambion), treated with DNase I (Ambion), and reverse-transcribed to cDNA (Quanta Bioscience). Endogenous expression of *OCT4, SOX2, KLF4, c-MYC, DNMT3B, NANOG,* and *GDF3* was tested by quantitative PCR (qPCR), and results were normalized on *ACTB* and parental fibroblast lines as described previously (3). qPCR data were analyzed with the 7500 System SDS software version 1.4 (Applied Biosystems).

Mutation Analysis in Fibroblasts and iPSCs. Genomic DNA was isolated using the QiAMP DNA kit (Qiagen), and genomic sequences of 400–600 bp around the reported mutations were amplified by PCR and purified using the QIAquick PCR purification kit (Qiagen). Purified DNA samples were sequenced by a core facility (Eton Bioscience) and analyzed with Sequence Scanner v1.0 (Applied Biosystems).

Karyotyping, G-Banding, and FISH Analysis. Karyotyping, G-banding, and FISH on fibroblasts and iPSCs were conducted by Cell Line Genetics. Parental fibroblast lines with LIG4 and DNA-protein kinase catalytic subunit (PKcs) mutations, their corresponding *coLIG4*- and mock-transduced cell lines, and two to eight clones per each iPSC line were tested for karyotypic abnormalities by G-banding. Parent and complemented fibroblast lines from 411BR were additionally analyzed for presence of the translocation *t*(3;8) by FISH using fluorochrome-conjugated probes specific for DNA sequences mapping at 8p23.2 and 8q24.3.

Irradiation, Immunocytochemistry, and Confocal Microscopy. Fibroblasts and iPSCs were grown to confluency on coverslips (VWR), irradiated with 5 and 0.5–2 Gy, respectively, and fixed with 4% (wt/vol) paraformaldehyde (PFA) for 30 min at room temperature, followed by ice cold methanol at −20 °C for at least 10 min at the given time points. Unirradiated cells were fixed at first time points to serve as controls. After permeabilization with 0.5% Triton/PBS (Roche), cells were stained for anti-phospho Ser-139 Histone H2AX (mouse, monoclonal, clone JBW301, 05-636; Millipore) and RAD51 (Ab-1 rabbit polyclonal, PC130; Calbiochem) or CENP-F (rabbit polyclonal, ab5; Abcam), respectively, in goat serum (Jackson ImmunoResearch), and subsequently with Alexa fluor anti-mouse/anti-rabbit 488/594 antibodies (Invitrogen). Coverslips were placed on slides (VWR) with DAPI-containing mounting media (Vector Laboratories).

Hematopoietic Differentiation of iPSCs Toward the Myeloid Lineage. Development of embryoid bodies (EBs) was performed as previously described (4). IPSCs were expanded in coculture with mouse embryonic fibroblasts (MEFs), dissociated with collagenase IV (STEMCELL), and cultured on low attachment plates (Corning) in the presence of IL-3, IL-6, human stem cell factor (hSCF), human Flt-3 ligand (hFlt-3L) (all purchased from Prepotech), and human bone morphogenetic protein-4 (BMP-4) and G-CSF (R&D Systems). After 2 wk, EBs were dissociated into single cells using collagenase B (Roche) and cell dissociation solution (Invitrogen) and were cultured in MethoCult H4434 (STEMCELL) for an additional 14 d.

Primer Sequences

Generation of LIG4 complementation vector.

Vector cloning	Forward primer	Reverse primer
Codon-optimized *LIG4*/vector backbone	CTGAGTCGGCCGGTGG-CCACCATGGCCGC	GGAGAGGGGCGGATCTCAGATC-AGGTACTGGTTCTCTTCC

qPCR of pluripotency-associated genes in generated iPSCs. Primer sequences used for amplification of *SOX2, KLF4, c-MYC, NANOG,* and *GDF3* have been published before (41). Primer sequences for *DNMT3B* and *OCT4* were obtained from Dr. Axel Schambach.

Gene	Forward primer	Reverse primer
DNMT3B	ATAAGTCGAAGGTGCGTCGT	GGCAACATCTGAAGCCATTT
OCT4	CCTCACTTCACTGCACTGTA	CAGGTTTTCTTTCCCTAGCT

qPCR of codon-optimized LIG4 and EGFP expression in complemented fibroblasts and iPSCs.

Gene	Forward primer	Reverse primer
co*LIG4*	TCTATCGCCAGCAACAAC	AACACGCTGAAGATGGTCT
EGFP	ACCATCTTCTTCAAGGACGA	CGTTGTGGCTGTTGTAGTTG

Mutation analysis in DCLRE1C, PRKDC, and LIG4. Mutations reported in the patients were confirmed in generated iPSC lines by sequencing of 400–600 bp around the mutation; in the case of large deletions (lines F04415 and NM720), whole exons were amplified.

Cell line (gene)	Mutation/exon	Forward primer	Reverse primer
F04415 (*DCLRE1C*)	Exon 1	GGACTTGGGATGGCGGCGC	GGGACAAGGCGTGTGCTG
F04415 (*DCLRE1C*)	Exon 2	TTCCTTGCAGAAGAAAGAGC	GAGTGTATTTGGTAGGATTATAT
F04415 (*DCLRE1C*)	Exon 3	AGATTTTGTGCCAGCGTAATTT	TGAACTCCTTGACCTCAAGAG
F04415 (*DCLRE1C*)	Exon 4	GTTTATATAGAGAGTCTGAAACT	AAGATTGAGGGTATAGCTGAG
F96224 (*DCLRE1C*)	L70del	CTGGTCTCAAATGAAATTATTAGATG	ATTAATGTGGATAACTGAAGTATGTTACA
F96224 (*DCLRE1C*)	G126D	TACTGTGGAATTCCATTACCATTG	CAGCAAGACTCCATTTCACAAA
ID177 (*PRKDC*)	L3062R	GAAACATATCTACCTTACATGATCC	CTGCATAAAACTCTGAATGC
NM720 (*PRKDC*)	A3574V	ACCTCATCTCTTGATAGAATAGATGC	TCTGCTATCAAAATAGCAGGCT
NM720 (*PRKDC*)	Exon 15–17	GCCCTGAGTCTGAATCTGAA	GCAAAATTCCACCAGGTTAAT
HYGM022 (*LIG4*)	K449Q	TTCCAGGTAGAATAGAAATAGTGC	CAATTCGTGGAAAACGC
HYGM022 (*LIG4*)	R814X	GCCCGTGAATATGATTGC	CTGGTTTTCTTCTTGTAATTCACAC
F07614/411BR (*LIG4*)	A3V, T9I	TAGGGTTGGAGCAAAACAGT	ATGAGTTCCAGTGGGTGTTC
F07614/411BR (*LIG4*)	R278H	GCACATTGAGAAGGATATG	CTCTATCCATCTACAAGCC
SCID072 (*LIG4*)	K424fs	TGGTAGAGGATTCTGATCTGC	CAATTCGTGGAAAACGC
SCID072 (*LIG4*)	Q558P	CTCCTGGTGAGAAGCCATC	AGTAAGGTTAGGTGCTTTTAAGTGC

1. Warlich E, et al. (2011) Lentiviral vector design and imaging approaches to visualize the early stages of cellular reprogramming. *Molec Therap* 19(4):782–789.
2. Miller JD, Schlaeger TM (2011) Generation of induced pluripotent stem cell lines from human fibroblasts via retroviral gene transfer. *Methods Mol Biol* 767:55–65.
3. Pessach IM, et al. (2011) Induced pluripotent stem cells: A novel frontier in the study of human primary immunodeficiencies. *J Allergy Clin Immunol* 127(6):1400–1407.
4. Cerdan C, Hong SH, Bhatia M (2007) Formation and hematopoietic differentiation of human embryoid bodies by suspension and hanging drop cultures. *Curr Protoc Stem Cell Biol* Chapter 1:Unit 1D 2.

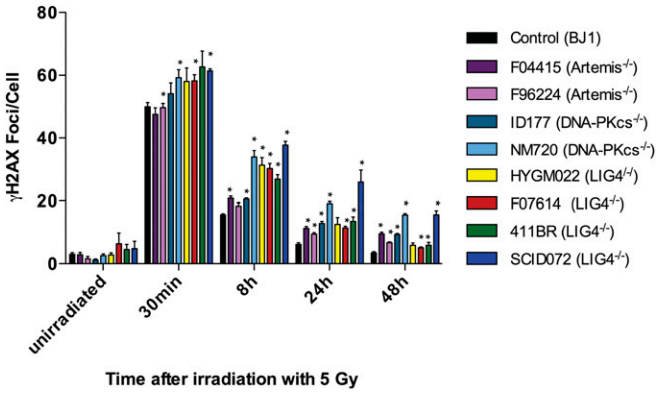

Fig. S1. Sensitivity to ionizing irradiation in Artemis-, DNA-PKcs–, and LIG4-mutated fibroblasts. Fibroblast lines from a healthy control and from patients with indicated NHEJ defects were irradiated with 5 Gy. Nuclear γH2AX foci formation was assessed by immunofluorescence staining and confocal microscopy in at least 50 cells per cell line at each time point. The mean number of γH2AX foci per cell at each time point was counted in three independent experiments. Results are expressed as mean with + SEM of γH2AX foci numbers per cell (*$P \leq 0.05$).

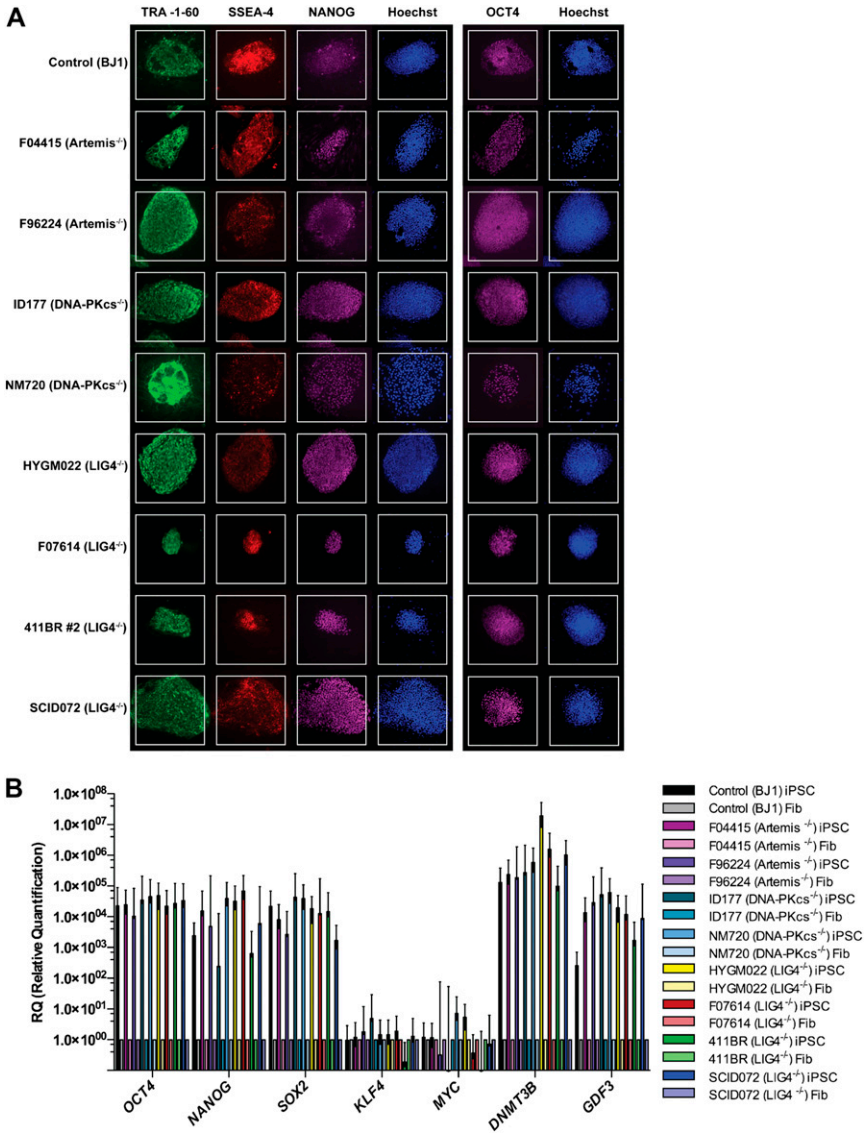

Fig. S2. Characterization of NHEJ-deficient iPSC lines. (*A*) Expression of pluripotency markers TRA-1–60, SSEA-4, NANOG, and OCT4 was assessed by immunofluorescence staining. The staining was done in two panels; for each panel, a single iPSC colony was imaged in all different channels. (*B*) RNA was isolated from all generated iPSC lines, and expression of endogenous genes associated with pluripotency (*OCT4*, *NANOG*, *SOX2*, *DNMT3B*, *GDF3)* and reprogramming *(KLF4, cMYC)* was analyzed by qPCR. Data were normalized on parental fibroblast lines and β actin (*ACTB*) was used as endogenous control. Results are expressed as mean value ± SEs.

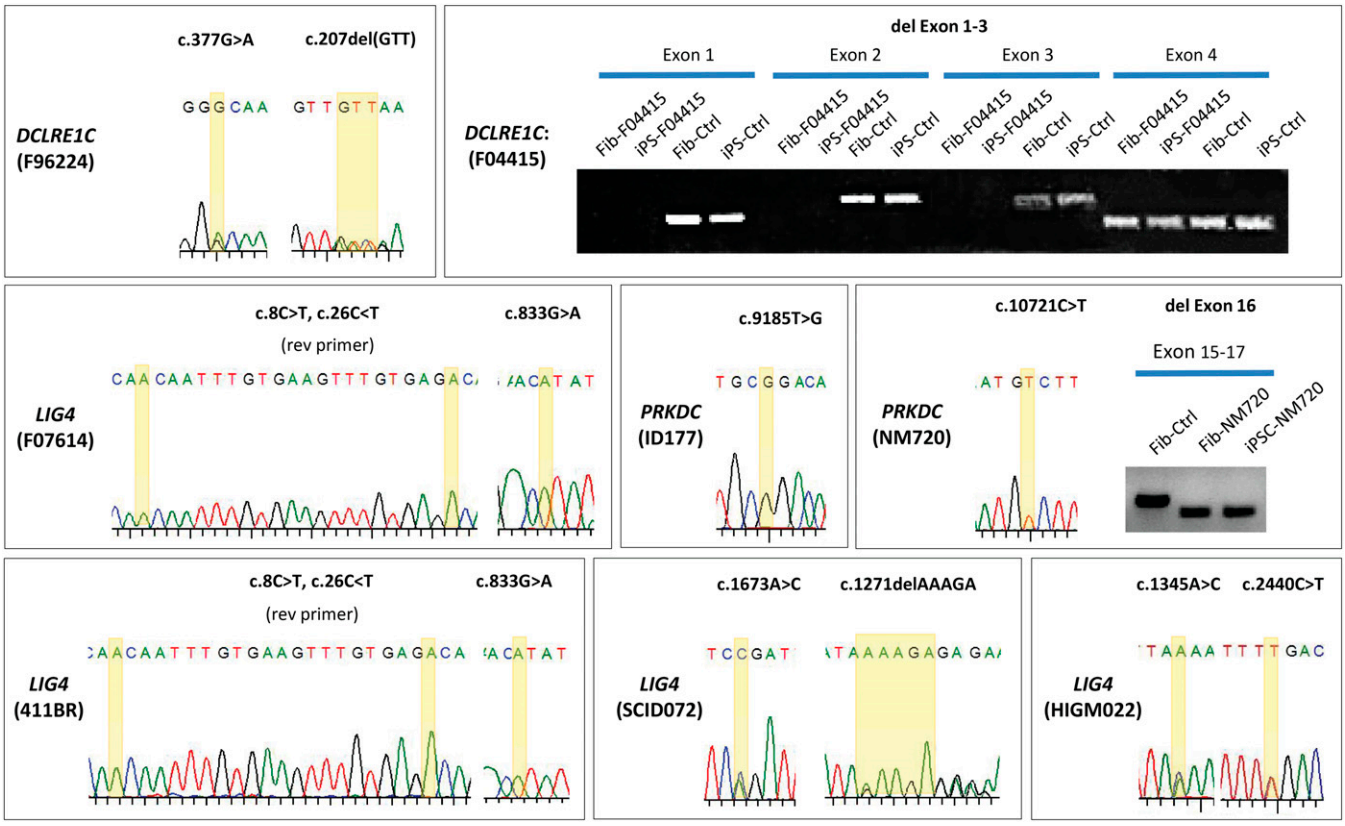

Fig. S3. Mutations in NHEJ-deficient iPSC lines. Genomic DNA was isolated from all iPSC lines used in this study. Mutations (marked in yellow) in *DCLRE1C*, *PRKDC,* and *LIG4* genes were confirmed by Sanger sequencing. Deletions of exons 1–3 of the *DCLRE1C* gene in the cell line F04415 and of exon 16 in the *PRKDC* gene in the cell line NM720 were analyzed by PCR.

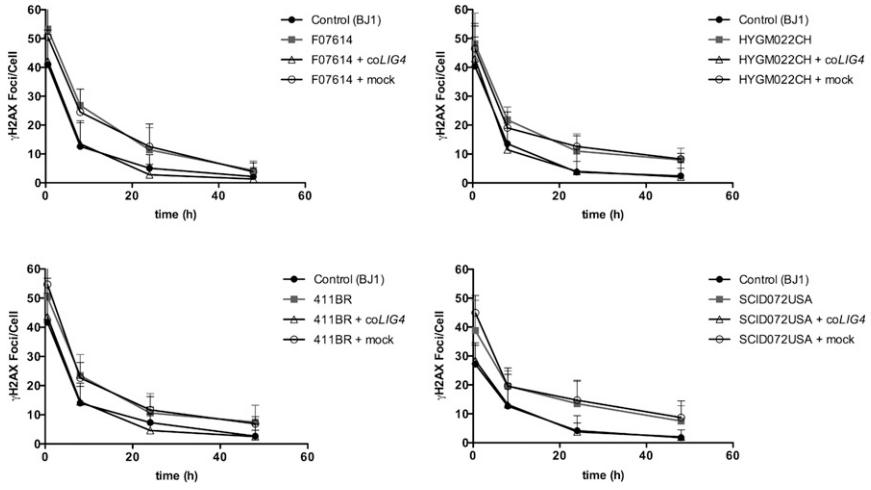

Fig. S4. Genetic complementation restores radiosensitivity in LIG4-deficient fibroblasts. LIG4-deficient fibroblast lines were transduced with codon-optimized *LIG4* (co*LIG4*) and mock lentiviral vectors. LIG4-complemented, mock-transduced, as well as control and the parental fibroblast lines were irradiated with 5 Gy, fixed at indicated time points, and analyzed for γH2AX foci formation. Mean numbers of γH2AX foci per cell (+SD) are shown for indicated cell lines. Significant difference ($P \leq 0.05$) was observed for all LIG4-complemented lines tested vs. parental and mock-transduced cell lines.

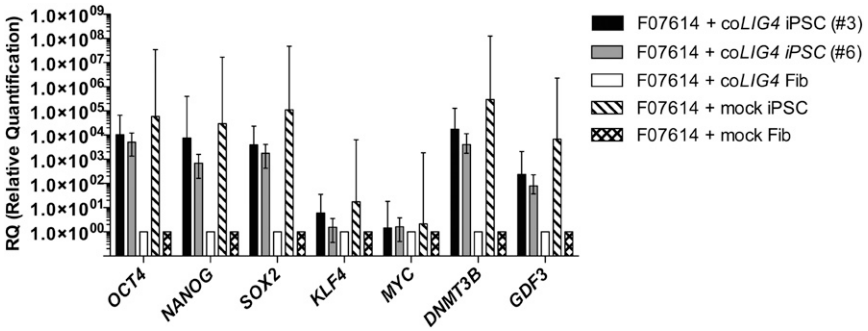

Fig. S5. Characterization of LIG4-complemented iPSC lines. qPCR analysis of expression of pluripotency related genes was performed on RNA isolated from LIG4-complemented and mock-transduced F07614 iPSCs. Values were normalized on parental complemented fibroblast lines, with *ACTB* expression as endogenous housekeeping gene control. Results are expressed as mean value ± SE.

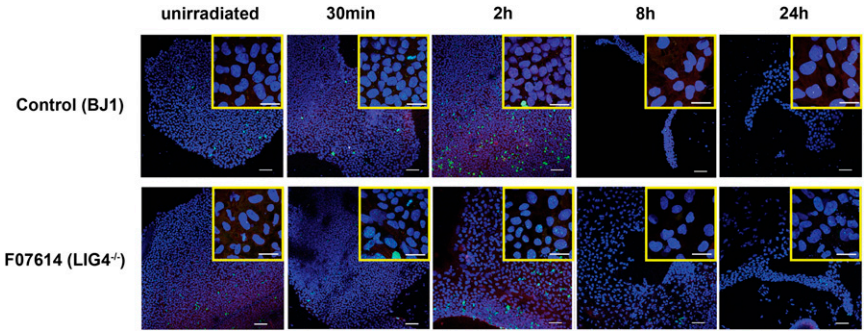

Fig. S6. Apoptosis in iPSCs in response to ionizing irradiation (IR). Induced pluripotent stem cells are extremely sensitive to IR and respond to a dosage as low as 1 Gy with apoptosis. Confocal images of control (BJ1) and LIG4-deficient (F07614) iPSC colonies are shown at indicated time points after IR with 1 Gy in extensive focus (10× and 60× objective). Channels for DAPI, Alexa fluor 488 (γH2AX), and Alexa fluor 594 (RAD51) are merged. (Scale bars, 50 μm.)

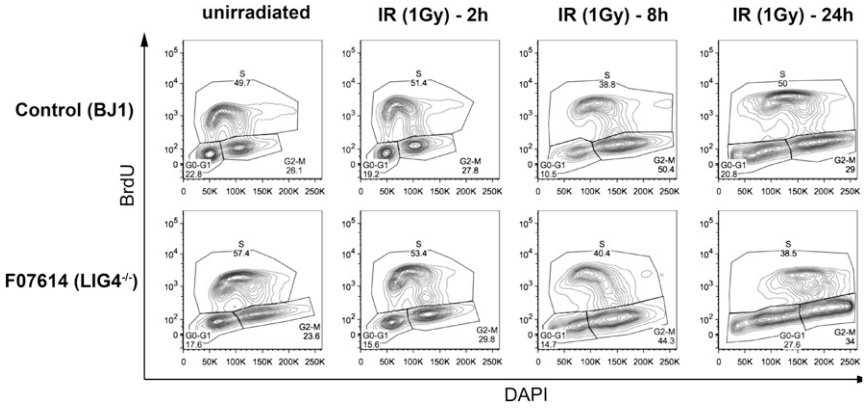

Fig. S7. Cell cycle of unsynchronized NHEJ-deficient iPSCs in response to IR. Nonsynchronized control (BJ1) and LIG4-deficient (F07614) iPSCs were irradiated with 1 Gy, pulsed with 10 μM BrdU for 45 min, and fixed at given time points. Nonirradiated cells were used as a control. Cell cycle was analyzed by flow cytometry after staining for BrdU and DAPI. After exclusion of dead and hypodiploid cells, proportions of cells in G0/G1, S, and G2/M phases of the cell cycle were assessed by gating as shown.

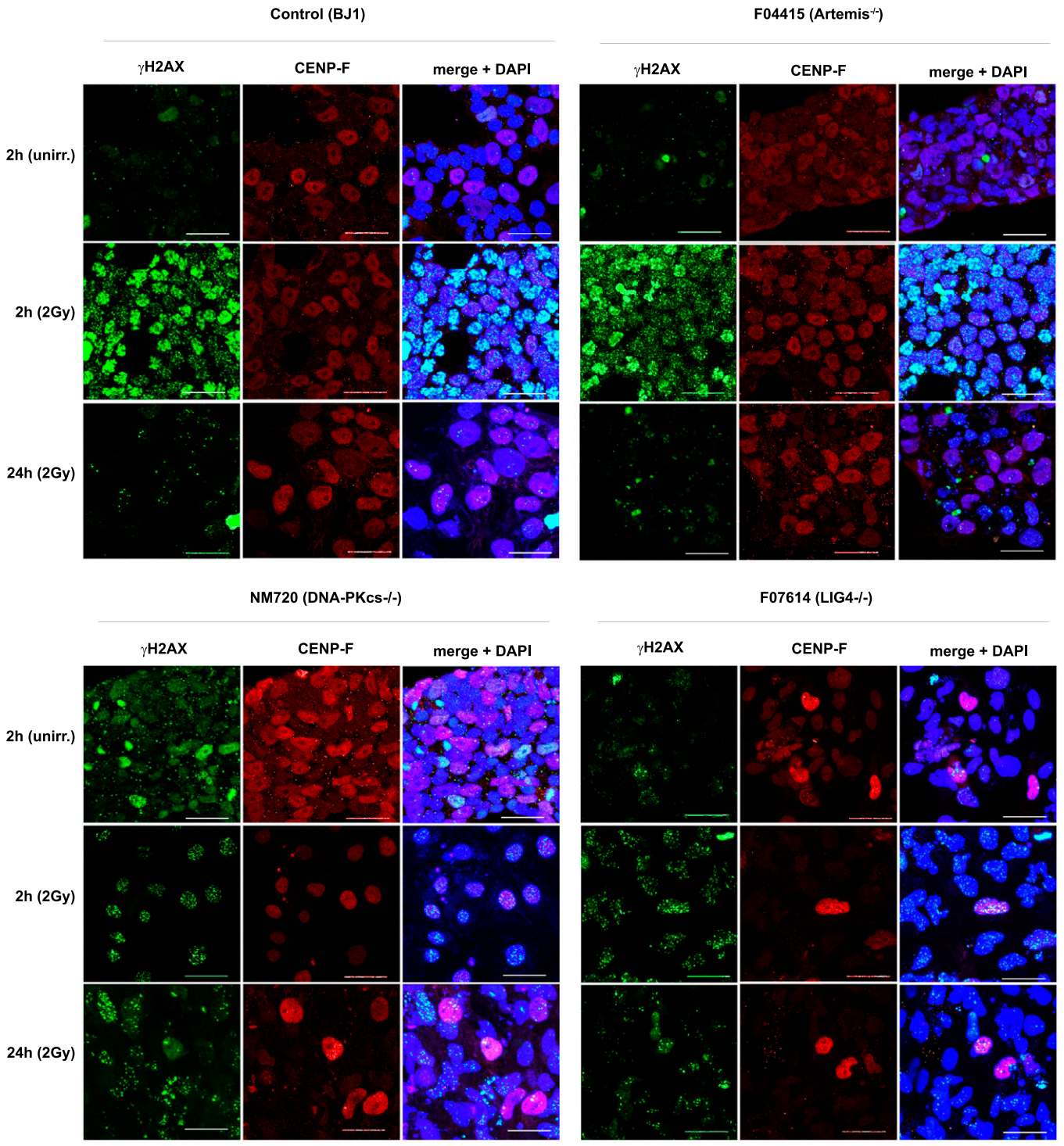

Fig. S8. Immunofluorescence staining of synchronized and irradiated iPSCs. Synchronized iPSC lines were irradiated (2 Gy) and fixed at 2, 4, 8, and 24 h. Nonirradiated cells 2 h after release from nocodazole treatment served as controls. Nuclear γH2AX foci formation and CENP-F expression were analyzed by coimmunostaining and confocal microscopy in at least 100 cells per cell line and time point. Identifications of cells in different phases of the cell cycle was determined by CENP-F expression (G0/G1: CENP-F^{-}; G2/M: CENP-F^{+}). Representative confocal images are shown for unirradiated cells and 2 and 24 h after exposure to IR. (Scale bars, 50 μm.)

Table S1. Karyotype and reprogramming efficiency in fibroblast and iPSC lines

Cell lines	Karyotype of fibroblasts [number of cells analyzed]	Karyotype of iPSCs [number of cells analyzed]	LIG4 complemented cell lines	Karyotype of LIG4 complemented fibroblasts [number of cells analyzed]	Karyotype of LIG4 complemented iPSCs [number of cells analyzed]
Control (BJ1)	Not done	#10: 46,XY [19] #12: 46,XY [20]			
F04415	Not done	#3: 46,XX [19] #6: 46,XX [20] #12: 46,XX [19]			
F96224	Not done	#2: 46,XY [20] #12: 46,XY [20]			
ID177	46,XX [20]	#3: 46,XX [19] #6: 46,XX [20]			
NM720	46,XY [19]	#2: 46,XY [20] #9: 46,XY [19] #12: 46,XY [19]			
HYGM022	46,XX [20]	#1–8: 46,XX [20]	HYGM022 + LIG4	46,XX [11]	No iPSC obtained
			HYGM022 + mock	No mitoses (senescence)	No iPSC obtained
F07614	46,XX [50]	#1: 46,XX [20]	F07614 + LIG4	46,XX [41]	#3: 46,XX [20]
				46,XXdel(14)(q24) [7]	#6: 46,XX [21]
			F07614 + mock	46,XX [43]	#4: 46,XX [20]
				46,XX del(14)(q24) [2]	#6: 46,XX [20]
411BR*	46,XY [29]	#1: 46,XY,*t*(3;8)(q29;q24.22), *t* (1;4)(q32.3;q27) [19]	411BR + LIG4	46,XY,*t*(3;8)(q29;q24.22) [19]	#6: 46,XY, *t*(3;8)(q29;q24.22) [20]
				46,XY,*t*(3;8)(q29;q24.22), *t*(7;13)(q21.2;q34) [1]	#8: 46,XY, *t*(3;8)(q29;q24.22) [19]
				FISH:	#10: 46,XY, *t*(3;8)(q29;q24.22) [20]
	46,XY,*t*(3;8)(q29;q24.22) [21]			46,XY [48]	
				46,XY, *t*(3;8)(q29;q24.22) [2]	
		#2: 46,XY,*t*(3;8)(q29;q24.22) [19]	411BR + mock	46,XY,*t*(3;8)(q29;q24.22) [18]	#4: 46,XY, *t* (2;3)(p15;p21.3) [19]
				46,XY,*t*(3;8)(q29;q24.22), *t* (1;20)(p10;p10) [1]	46,XY,*t* (2;3)(p15;p21.3), add(14)(q32) [1]
				FISH:	#5: 46,XY, *t* (2;3)(p15;p21.2) [19]
				46,XY [33]	#7: 46,XY [19]
				46,XY,*t*(3;8)(q29;q24.22) [17]	
SCID072	46,XY [49]	#1: 46,XY,add(14)(q32.3) [18]	SCID072 + LIG4	46,XY [47]	#1: 46,XY [20]
				46,XY,*t*(11;18)(p15.3;q21.1) [2],	#2: 46,XY,*t*(11;18)(p15.3;q21.1) [17], 46,XY,*t*(7;15)(q36;q22), *t*(11;18)(p15.3;q21.1) [1]
	46,XY,add(14)(q32.3) [1]			46,XY,*t*(10;19)(q23;p13.1) [1]	#3: 46,XY,*t*(3;5)(q26.2;q31) [19]
					#4: 46,XY [20]
			SCID072 + mock	46,XY [16]	No iPSC obtained
				46,XY,*t*(7;13)(q11.23;q32) [3]	
				46,XY,*t*(6;14)(q27;q11.2) [1]	

*411BR iPSCs #1 and #2 have been generated in two independent reprogramming experiments.