Lamin A/C depletion enhances DNA damage-induced stalled replication fork arrest

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Lamin A/C Depletion Enhances DNA Damage-Induced Stalled Replication Fork Arrest

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The human LMNA gene encodes the essential nuclear envelope proteins lamin A and C (lamin A/C). Mutations in LMNA result in altered nuclear morphology, but how this impacts the mechanisms that maintain genomic stability is unclear. Here, we report that lamin A/C-deficient cells have a normal response to ionizing radiation but are sensitive to agents that cause interstrand cross-links (ICLs) or replication stress. In response to treatment with ICL agents (cisplatin, camptothecin, and mitomycin), lamin A/C-deficient cells displayed normal γ-H2AX focus formation but a higher frequency of cells with delayed γ-H2AX removal, decreased recruitment of the FANCD2 repair factor, and a higher frequency of chromosome aberrations. Similarly, following hydroxyurea-induced replication stress, lamin A/C-deficient cells had an increased frequency of cells with delayed disappearance of γ-H2AX foci and defective repair factor recruitment (Mre11, CtIP, Rad51, RPA, and FANCD2). Replicative stress also resulted in a higher frequency of chromosomal aberrations as well as defective replication restart. Taken together, the data can be interpreted to suggest that lamin A/C has a role in the restart of stalled replication forks, a prerequisite for initiation of DNA damage repair by the homologous recombination pathway, which is intact in lamin A/C-deficient cells. We propose that lamin A/C is required for maintaining genomic stability following replication fork stalling, induced by either ICL damage or replicative stress, in order to facilitate fork regression prior to DNA damage repair.

Lamins are intermediate filament proteins that form a protein meshwork lining the inner nuclear membrane, where they contribute to maintaining the shape and mechanical stability of the nucleus (1). Lamin proteins interact with histone H2A (2, 3) and also form nucleoplasmic foci that perform dynamic organizational roles in the nucleus (4, 5). Human lamins A and C (lamin A/C) are generated from a single LMNA gene (Lmna in mice) by alternative splicing, and mutations that disrupt splicing are the basis for a variety of degenerative disorders, including premature aging syndromes and cancer. Mutations in the LMNA gene have also been linked to chromatin modifications that, when defective, are associated with altered DNA transcription, replication, and repair. About 200 disease-associated LMNA mutations have been identified (6), and the resulting laminopathies all are characterized by chromosomal aberrations (7, 8). Although lamins are implicated in chromatin organization, DNA replication, RNA polymerase II-dependent gene expression, and DNA damage response (DDR) (8–11), Lmna deletion in mice is not lethal (12, 13). However, cells from Lmna−/− mice do display loss of chromatin integrity with deformation or blebbing of the lamina and nuclear envelope (13). Chromatin changes related to loss of structural shape, in conjunction with transcription regulatory changes (12, 14, 15), can also alter the DDR, resulting in DNA damage accumulation (16). While chromatin changes have been linked with altered gene expression, altered expression of DDR-related genes due to lamin A/C deficiency have not yet been identified. We compared the gene expression profile between cells with and without Lmna and report here that loss of lamin A/C results in decreased cyclin D1 levels. In an examination of clonogenic survival and DNA damage response/repair, we found that cells deficient in lamin A/C have decreased survival, defective DNA damage response, and decreased restart of stalled replication forks after exposure to agents that cause interstrand cross-links (ICLs), DNA adducts, and replication stress.

MATERIALS AND METHODS

Colony-forming assay and chromosomal aberration analysis. Lmna+/+, Lmna−/−, 293, MCF7, and GM5849 cells were maintained and transfected with plasmids as described previously (17, 18). A cDNA fragment encoding wild-type lamin A was cloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) as described previously (17, 19). Small interfering RNAs (siRNAs) for human LMNA, cyclin D1, RAD51, ORCA1, polymerase η (Pol η), and control luciferase (Luc) were obtained from Dharmacon Research (Lafayette, CO). RNA interference (RNAi) treatment of cell lines was performed as described previously (17, 19, 20). Cells were used 72 h after transfection for all experimental purposes. Colony-forming assays and analysis of metaphase spreads to mea-
sure chromosomal aberrations were carried out as described previously (20).

**Microirradiation.** Lmna<sup>+/+</sup> and Lmna<sup>−/−</sup> cells expressing yellow fluorescent protein (YFP)-labeled polymerase η were grown on cover slips. Microirradiation and quantification of polymerase η signal at different time points postirradiation was done as described previously (18).

**Cell cycle analysis.** Murine embryonic fibroblasts (MEFs) were synchronized by culturing in Dulbecco’s modified Eagle medium (DMEM) containing 0.1% fetal calf serum (FCS) for 72 h and then stimulated with DMEM supplemented with 10% FCS at various time points. After serum stimulation, cells were harvested by trypsinization and processed for fluorescence-activated cell sorter (FACS) analysis by a previously described procedure (21, 22). To determine the frequency of S-phase cells after nocodazole treatment, Lmna<sup>+/+</sup> and Lmna<sup>−/−</sup> cells were treated for 20 h with nocodazole to arrest them in G<sub>2</sub>/M, and after release cells were harvested at the indicated time points, stained with propidium iodide (PI), and subjected to FACS analysis to determine percentage of cells entering G<sub>1</sub> phase.

**Immunofluorescence microscopy.** Cell culture in chamber slides, fixation, and immunostaining were done as previously described (23–25). For depletion of lamin A/C, 293 cells were transfected with the specific siRNA and allowed to grow for 48 h before treatment with the indicated DNA-damaging agent or mock treatment. Cells then were treated with an extraction buffer for 5 min before fixation in 4% paraformaldehyde (PFA). A standard procedure for capturing fluorescent images of foci was followed (26). Sections through nuclei were captured, and the images were obtained by projection of the individual sections as recently described (27). The results shown are from three to four independent experiments.

**Expression profiling.** A previously described method was used for microarray analysis of gene expression (28). Total RNA was isolated from cells using the RNeasy kit (Qiagen), and gene expression analyses were performed using the Illumina mouse gene expression array (MouseWG-6 v2). The array images were processed using Illumina Genome Studio per the manufacturer’s instructions, and the intensity values were background subtracted and quantile normalized. The differential gene expression was detected by fold change cutoff (≥2 or ≤-2), and the gene list was uploaded to the Ingenuity pathway analysis (IPA) program for cellular function enrichment and biological network analyses.

**DNA replication restart assay.** Exponentially growing cells were pulsed with 50 mM 5-iododeoxyuridine (IdU) for 20 min, washed three times with phosphate-buffered saline (PBS), treated with 2 mM hydroxyurea (HU) for the indicated intervals, washed three times with PBS, incubated in fresh medium containing with 50 mM 5-chlorodeoxyuridine (CldU) for 20 min, and then washed three times in PBS. DNA fiber spreads were made by a modified procedure described previously (29). Briefly, cells labeled with IdU and CldU were mixed with unlabelled cells in a ratio of 1:10, and 2-μl cell suspensions were dropped onto a glass slide and then mixed with a 20-μl hypotonic lysis solution (10 mM Tris-HCl, pH 7.4, 2.5 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5% Nonidet P-40) for 8 min. Air-dried slides were fixed, washed with 1× PBS, blocked with 5% bovine serum albumin (BSA) for 15 min, and incubated with primary antibodies against IdU and CldU (rat monoclonal antibody [Mab] anti-IdU [1:150 dilution; Abcam] and mouse Mab anti-CldU [1:150 dilution; BD]) and secondary antibodies (anti-rat Alexa Fluor 488-conjugated [1:150 dilution] and anti-mouse Alexa Fluor 568-conjugated [1:200 dilution] antibodies) for 1 h each. Slides were washed with 1× PBS with 0.1% Triton X-100 and mounted with Vectashield mounting medium without 4′,6-diamidino-2-phenylindole (DAPI). Image J software was used to analyze the DNA fibers.

**Determination of collapsed replication fork.** Mouse embryonic fibroblasts were labeled with 1 μM 5-ethyl-2′-deoxyuridine (EdU) for 30 min to stain S-phase cells, washed twice with 1× PBS, incubated in medium with 0.1 mM HU for 12 h, washed again with 1× PBS to remove HU, and incubated in fresh medium for 8 h after HU treatment. Cells were stained for γ-H2AX and EdU, and EdU-positive G<sub>2</sub>-phase cells (determined by their DAPI intensity) were evaluated. First, we scored only large γ-H2AX foci, which only arise after long HU treatment times and thus are supposed to represent collapsed replication forks (30). Second, we scored the percentage of EdU-positive cells, which migrated into G<sub>1</sub> during the 8-h time period after HU treatment. We found delayed progression into G<sub>1</sub> phase in Lmna<sup>−/−</sup> cells. That is, despite the lower focus numbers, the Lmna<sup>−/−</sup> cells did not readily progress into G<sub>1</sub>, again consistent with the model that the lamin A/C-deficient cell is arrested in S phase because fork collapse is impaired.

**MOF retention in Lmna<sup>+/+</sup> and Lmna<sup>−/−</sup> cells.** Cells growing in exponential phase were irradiated and fixed with 4% formaldehyde at different times postirradiation, and total DNA was coimmunoprecipitated with MOF antibody after in vitro cross-linking by using the standard procedure described previously (31, 32). Immunoprecipitated DNA was purified by the phenol-chloroform procedure (33). DNA was quantified with a NanoDrop 2000 spectrometer (Thermo Scientific), and the amount of DNA retained by MOF is presented in arbitrary relative units of retention (MOF retention).

**Telomeric circle detection and strand-specific FISH.** Genomic DNA from exponentially growing cells was isolated, digested by standard protocols (31, 32), and fractionated on a 0.7% agarose gel containing 0.1 μg/ml ethidium bromide in 1× Tris-acetate-EDTA at −2 V/cm overnight. Neutral-neutral two-dimensional (2D) gel electrophoresis was performed according to the established protocols (34), with modifications as described previously (35). Strand-specific chromosome orientation fluorescent in situ hybridization (CO-FISH) was performed by the described procedure (26, 36).

**RESULTS**

**Effect of lamin A/C depletion on gene expression.** To elucidate the relationship between lamin A/C and genomic stability, a lamin A/C functional interaction network (Fig. 1A) based on the literature was first generated from IPA. Relative mRNA expression data were obtained by a microarray analysis of Lmna<sup>−/−</sup> versus Lmna<sup>+/+</sup> mouse embryonic fibroblast (MEF) RNA (Fig. 1B and C), and the up- and downregulated genes were overlaid on the IPA network (Fig. 1A). Lamin A/C clearly impacts multiple cellular functions (major altered functions are shown in Fig. 1A). The complete microarray data are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38777. We have summarized the top five genes that are up- or downregulated in Lmna<sup>−/−</sup> cells compared to Lmna<sup>+/+</sup> cells (Fig. 1C) in the following categories.

**Cancer-related genes.** Upregulated cancer-related genes are those for prostaglandin 12 (prostacyclin) synthase (PTGIS), keratin 14 (KRT14), urorplakin 3B (UPK3B), keratin 8 (KRT8), and basonuculin 1 (BNC1). Downregulated genes are those for CYP7B1, collagen type VI alpha 3 (COL6A3), alpha interferon-inducible protein 27-like 2 (IFI27L2), aquaporin 1 (Colton blood group) (AQP1), and fibroblast growth factor 7 (FGF7).

**Proliferation and growth genes.** Upregulated proliferation and growth genes are the transferrin (TF), KRT8, basonuculin 1 (BNC1), homeobox B7 (HOXB7), and tripartite motif-containing 25 (TRIM25) genes. Downregulated genes are those for cytochrome P450 family 7 subfamily B polypeptide 1 (CYP7B1), COL6A3, dickkopf homolog 3 (Xenopus laevis) (DKK3) FGF7, and lamin A/C (LMNA).

**Cell cycle-related genes.** Upregulated cell cycle-related genes...
FIG 1 Functions of lamin A/C. (A) Ingenuity pathway analysis of lamin A/C based on mRNA microarray expression data from comparisons of mRNA from Lmna<sup>−/−</sup> to that of Lmna<sup>+/−</sup> cells. Pink indicates upregulated and green downregulated genes. The top 12 relevant biological functions and disease associations from the analysis are shown on both sides of the figure. (B) Comparison of mRNA expression status between Lmna<sup>+/−</sup> and Lmna<sup>−/−</sup> cells. The expression is organized into eight major groups involved in cellular metabolism. The eight major groups are cancer, proliferation and growth, cell cycle, cell death, signaling ligands and NF-κB, lipid metabolism, interferon signaling, and glutathione metabolism. (C) mRNA expression is organized from highest to lowest in Lmna<sup>+/−</sup> cells compared to Lmna<sup>−/−</sup> cells. Details of the expression levels are available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38777. (D) Western blot showing cyclin D1 protein levels in Lmna<sup>+/−</sup> and Lmna<sup>−/−</sup> cells. (E) Western blot showing lamin A/C knockdown by LMNA-specific siRNA. (F) Western blot showing decreased cyclin D1 levels in 293 cells treated with lamin A/C-specific siRNA. (G) Coimmunoprecipitation of endogenous lamin A/C and cyclin D with anti-lamin A/C antibody detected by immunoblotting.
FIG 2 Ionizing radiation response in cells with and without lamin A/C. (A) Clonogenic survival of Lmna+/+ and Lmna−/− mouse cells after exposure to increasing radiation doses. (B) Western blot showing depletion of cyclin D1 by specific siRNA in Lmna−/− and Lmna+/+ cells. (C) Clonogenic survival of cells with and without cyclin D1 knockdown after exposure to graded doses of IR. (D) Exponentially growing cells were irradiated with 2 Gy, and the appearance/disappearance of γ-H2AX foci was determined by immunostaining. (E) Lmna+/+ and Lmna−/− cells were irradiated with 6 Gy, and 53BP1 focus formation was quantified by immunostaining at different time points postirradiation. (F) Exponential-phase Lmna+/+ and Lmna−/− cells were irradiated with different IR doses, and RAD51 focus formation was quantified 4 and 8 h postirradiation. (G) Ionizing radiation-induced phosphorylation of ATM Ser1981, detected by immunoblotting, in exponentially growing Lmna+/+ and Lmna−/− cells after irradiation with 5 Gy. (H) Ionizing radiation-induced phosphorylation of DNA-PK catalytic subunit (DNA-PKcs) in 293 cells with and without siRNA depletion of lamin A/C detected by DNA-PKcs and phospho-Ser2056 immunoblotting. Cells were irradiated with 10 Gy and collected at different time points postirradiation for analysis. (I) Chromosomal aberrations in Lmna+/+ and Lmna−/− cells after IR exposure. For analysis of G1-phase aberrations, cells were irradiated (3 Gy), incubated for 12 h, and then treated for 3 h with colcemid before collecting metaphases for scoring. Categories of asymmetric chromosome aberrations scored included dicentrics, centric rings, interstitial deletionsacentric rings, and terminal deletions. For S-phase-specific aberrations, cells were irradiated with 2 Gy and incubated for 6 h, and metaphases were harvested after 3 h of colcemid treatment. For G2-type chromosome aberrations, exponential-phase cells were irradiated (1 Gy) and incubated for 1 h, followed by 3 h of colcemid treatment to collect metaphases.
are those for transferrin (TF), TRIM25, insulin-like growth factor binding protein 3 (IGFBP3), bone morphogenetic protein 4 (BMP4), and paired-like homeodomain 2 (PITX2). Downregulated genes are those for interleukin 7 (IL-7), interferon regulatory factor 7 (IRF7), lymphocyte antigen 6 complex, locus C1 (LY6C1), FGF7, and LMNA.

Cell death-related genes. Upregulated cell death-related genes are those for PTGIS, TF, KRT8, IGFBP3, and IGFBP6. Downregulated genes are those for matrix Glα protein (MGP), ubiquitin specific peptidase 18 (USP18), necdin homolog (mouse) (NDN), DKK3, and FGF7.

Signal ligand and NF-κB genes. Upregulated signal ligand and NF-κB genes are those for IGFBP3, insulin-like growth factor 1 (somatomedin C) (IGF1), intercellular adhesion molecule 1 (ICAM1), vascular endothelial growth factor C (VEGFC), and nuclear factor of kappa light polypeptide gene enhancer in B cells 2 (p49/p100) (NFKB2). Downregulated genes are those for myosin light chain 6B, alkali, smooth muscle and nonmuscle (MYL6B); fibroblast growth factor receptor 1 (FGFR1); signal transducer and activator of transcription 1 (STAT1); IGFBP5; and chemokine (C-C motif) ligand 5 (CCL5).

Lipid metabolism genes. Upregulated lipid metabolism genes
are those for glutathione S-transferase alpha 3 (GSTA3), glutathione peroxidase 7 (GPX7), paternally expressed 3 (PEG3), COP9 constitutive photomorphogenic homolog subunit 8 (Arabidopsis) (COP8), and glutathione S-transferase kappa 1 (GSTK1). Downregulated genes are those for are corneodesmosin (CDSN), microsomal glutathione S-transferase 3 (MGST3), adrenomedullin (ADM), glutathione S-transferase alpha 5 (GSTA5), and LMNA.

**Glutathione metabolism genes.** Upreregulated glutathione metabolism genes are those for GSTA3, GPX7, GSTK1, GSTA4, and MGST3. Downregulated genes are those for acyl coenzyme A synthetase short-chain family member 2 (ACSS2), glutathione S-transferase theta 3 (GSTT3), glutaredoxin (thioltransferase) (GLRX), MGST3, and GSTA5.

**Interferon signaling genes.** The upregulated interferon signaling gene is that for Janus kinase 2 (JAK2) (only one gene is upregulated in this category). Downregulated genes are those for STAT2; transporter 1, ATP-binding cassette, subfamily B (MDR/TAP) (TAP1); myxovirus (influenza virus) resistance 1, interferon-inducible protein 78 (mouse) (MX1); 2',5'-oligoadenylate synthetase 1 (OAS1); and IFIT3.

**Ubiquitin pathway genes.** Lmna−/− cells have 230-fold decreased USP18 levels compared to those of Lmna+/+ cells.

Specifically, cyclin D1 mRNA was significantly downregulated in Lmna−/− cells (Fig. 1C), and consequently cyclin D1 protein was barely detectable in Lmna−/− cells (Fig. 1D). Ectopic lamin A expression in Lmna−/− cells restored cyclin D1 to the levels observed in Lmna+/+ cells (Fig. 1D). Similarly, in human 293 cells, siRNA depletion of lamin A/C (Fig. 1E) greatly reduced cyclin D1 levels (Fig. 1F). In addition, cyclin D1 coimmunoprecipitates with lamin A, suggesting the two proteins interact either directly or indirectly (Fig. 1G).

**Role of lamin A/C on ionizing radiation response.** Jirawatnotai et al. (37) reported that cyclin D1 plays a role in ionizing radiation response and DNA repair, and we observed that exponentially growing Lmna−/− MEFs and human 293 cells depleted for lamin A/C had marginally higher survival postirradiation than the respective control cells (Fig. 2A and data not shown); however, the differences were not statistically significant. This was unexpected, since lamin A/C-deficient cells have reduced levels of cyclin D1 (Fig. 1D and F). Cyclin D1 interacts with DNA repair proteins, and cyclin D1 depletion was previously shown to reduce postirradiation survival (37). Depletion of cyclin D1 from Lmna−/+/+ cells (Fig. 2B) did, however, reduce clonogenic survival postirradiation (Fig. 2C), in line with the previously published results, suggesting that lamin A/C depletion reverses the increase in ionizing radiation (IR)-induced cell death resulting from cyclin D1 depletion.

Consistent with the clonogenic survival results, comparison of Lmna−/− to Lmna+/+ exponential-phase cells following IR exposure did not reveal any significant difference in the DDR as determined by the appearance/disappearance kinetics of γ-H2AX foci (Fig. 2D). Similarly, 53BP1, Rad51, FANCID2, CtIP, and RAP80 focus formation in exponential-phase Lmna−/− or Lmna+/+ cells was nearly identical (Fig. 2E and F and data not shown). In addition, neither ATM nor DNA-dependent protein kinase (DNA-PK) phosphorylation following irradiation was altered by lamin A/C loss (Fig. 2G and H). Furthermore, when cells were irradiated in different phases of the cell cycle, the frequency of residual chromosome aberrations observed at metaphase was similar in

![FIG 4 Impaired DNA damage response in lamin A/C-depleted cells. (A to C) Cells with γ-H2AX foci after treatment with cisplatin (A), MMC (B), and camptothecin (C). (D) Cells with FANCID2 foci after cisplatin and MMC treatment. (E) Frequency of Lmna+/+ and Lmna−/− metaphases with chromosome aberrations after cisplatin treatment. (F) Frequency of 293 metaphases with and without lamin A/C with chromosome aberrations after cisplatin treatment. (G) Frequency of Lmna+/+ and Lmna−/− metaphases with chromosome aberrations after MMC treatment. *, P < 0.05; **, P < 0.01.
Lmna<sup>−/−</sup> and Lmna<sup>+/+</sup> cells (Fig. 2I). Lamin A/C therefore appears to play no role in the cellular response to ionizing radiation as determined by clonogenic cell survival, DNA damage signaling, or chromosome repair.

Response of lamin A/C-deficient cells to DNA cross-linking and adduct-inducing agents. The results described above indicate that IR-induced DDR in lamin A/C-deficient cells is largely unaltered. Therefore, we asked whether lamin A/C is involved in the repair of DNA intra- or interstrand cross-links (ICLs) or simple DNA adduct damage. ICLs create obstructions to fundamental DNA transactions and are repaired predominantly during S-phase, initiated by replication fork convergence at ICL sites (38). As measured by cell survival, Lmna<sup>−/−</sup> cells were more sensitive to intra- and interstrand cross-linking agents like cisplatin, mitomycin C (MMC), and formaldehyde (Fig. 3A, B, and C). The Lmna<sup>−/−</sup> cells were more sensitive to DNA topoisomerase inhibitors like camptothecin and VP16 (Fig. 3D and E) and thymidine dimer formation induced by UV (Fig. 3F). DNA monoalkylating drugs like N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (Fig. 3G) were also more lethal to Lmna<sup>−/−</sup> cells. In addition, we found that Lmna<sup>−/−</sup> cells were much more sensitive to replication stress induced by HU treatment than Lmna<sup>+/−</sup> cells, as determined by clonogenic assay (Fig. 3H and I). Depletion of lamin A/C from human 293 cells (Fig. 1F) also resulted in hypersensitivity for cell survival to HU (Fig. 3I), and these cells also had a higher frequency of cells with delayed disappearance of γ-H2AX foci compared to cells expressing lamin A/C (Fig. 4A to C and 5A).

FIG 5 Hydroxyurea treatment response in cells with and without lamin A/C. (A) Frequency of cells with γ-H2AX at different time points after treatment. (B) Lmna<sup>−/−</sup> and Lmna<sup>+/−</sup> cells stained for DNA with DAPI and γ-H2AX immunostaining. EdU-positive S/G<sub>2</sub>-phase cells (selected by DAPI staining) were selected only for γ-H2AX focus counting. (C) The number of larger γ-H2AX foci counted in late S/G<sub>2</sub>-phase cells was performed by using scatter blots obtained by scanning the slides for DAPI and EdU intensity. γ-H2AX foci were counted in ~40 cells per data point and experiment per slide. (D) Histogram showing percent EdU-positive G<sub>1</sub> cells. A total of 3,000 cells were counted, and the means from three experiments are plotted. *, P < 0.05; **, P < 0.01. (E to H) Frequency of cells with Mre11 (E), CtIP foci (F), RAD51 (G), RPA (H), and FANC2 (I) foci after HU treatment. (J) HU-treated lamin A/C-depleted 293 cells at metaphase with chromosome aberrations, including a triradial (arrow). (K) Histogram showing a comparison of chromosome aberrations per metaphase in Lmna<sup>−/−</sup> cells, Lmna<sup>+/−</sup> cells, and Lmna<sup>−/−</sup> cells with ectopic expression of lamin A.
Furthermore, FANCD2 focus induction after cisplatin or MMC treatment was strongly reduced in Lmna<sup>−/−</sup> cells compared to that in Lmna<sup>+/+</sup> cells (Fig. 4D). Both mouse (Fig. 4E) and human (Fig. 4F) lamin A/C-deficient cells displayed a higher frequency of metaphases with chromosome aberrations after cisplatin, mitomycin, or camptothecin treatment compared to cells with lamin A/C (Fig. 4E to G and data not shown). Treatment of Lmna<sup>−/−</sup> cells with HU also significantly delayed the disappearance of g-H2AX foci (Fig. 5A); reduced the number of large g-H2AX foci (Fig. 5B), which are an indication of collapsed forks (Fig. 5C); and delayed cell cycle progression (Fig. 5D). Consistent with the observation of abnormally sized g-H2AX foci, a significant defect in the recruitment of Mre11, CtIP, RAD51, RPA, and FANC2 into foci was observed in lamin A/C-deficient cells (Fig. 5E to I). In addition, lamin A/C-deficient cells had a high frequency of HU-induced chromosome aberrations, including triradials that could be suppressed by lamin A cDNA expression in Lmna<sup>−/−</sup> cells (Fig. 5I and K).

During repair of DNA strand cross-links, DNA polymerase η plays an important role in translesion DNA synthesis (39, 40). Cells deficient for either lamin A/C or polymerase η (Fig. 6A) had a higher frequency of MMC- and HU-induced chromosome aberrations than cells expressing lamin A/C, and an even higher level of aberrations was observed in doubly deficient cells (Fig. 6B and C). Furthermore, polymerase η recruitment to UV550-induced DNA damage sites was significantly delayed in Lmna<sup>−/−</sup> cells (Fig. 6D and E). Thus, lamin A/C-deficient cells have compromised DNA interstrand cross-link repair.

Role of lamin A/C in the restart of stalled replication forks.

To determine the mechanism by which lamin A/C deficiency results in defective ICL repair, we compared the restart of stalled replication forks in Lmna<sup>−/−</sup> and Lmna<sup>+/+</sup> cells by using the DNA fiber technique (41). We first determined the frequency of stalled replication forks and new origins. Cells were pulse-labeled with 5-iododeoxyuridine (IdU), treated with HU for different time periods to deplete the nucleotide pool, and then washed and pulse-labeled with 5-chlorodeoxyuridine (CldU) (Fig. 7A) as described previously (42, 43). Replication fork restart was quantified by determining the total number of replication tracks labeled with CldU (Fig. 7B). After removal of the HU block, contiguous IdU/CldU signal, indicating restarting forks, was rarely seen in Lmna<sup>−/−</sup> cells compared to Lmna<sup>+/+</sup> cells that readily resumed DNA synthesis (Fig. 7B to D). Thus, replication had not restarted at the stalled replication forks during the period analyzed in Lmna<sup>−/−</sup> cells. While analyzing DNA fibers (Fig. 7Aii), we found that the percentage of stalled forks in Lmna<sup>−/−</sup> cells after 1 or 21 h of HU treatment was higher than that of Lmna<sup>+/+</sup> cells (Fig. 7E and F). Ectopic expression of lamin A reduced stalled forks and...
increased new origins in Lmna<sup>−/−</sup> cells treated with HU (Fig. 7E and F). To test whether the failure to initiate new replication restart was due to differences in the median IdU track length between Lmna<sup>+/+</sup> and Lmna<sup>−/−</sup> cells, we measured IdU track length with and without treatment of HU for 5 h and found that in HU-treated Lmna<sup>+/+</sup> cells the IdU tract length is maintained, whereas in Lmna<sup>−/−</sup> cells IdU tract length is significantly reduced (Fig. 7G).

Cells deficient in lamin A/C have undetectable levels of cyclin D1 (Fig. 1D and F), which is associated with lamin A in wild-type cells, but treatment of Lmna<sup>+/+</sup> cells with HU decreases this interaction (Fig. 8A and B). To determine whether the enhanced replication fork stalling seen in Lmna<sup>−/−</sup> cells could be due to low levels of cyclin D1, we examined HU-treated Lmna<sup>−/−</sup> cells with and without ectopic expression of cyclin D1 for stalled forks as well as new origins. Depletion of cyclin D1 significantly increased the number of stalled forks and decreased new origins in Lmna<sup>−/−</sup> but not in Lmna<sup>+/+</sup> cells (Fig. 8C and D). Increased cyclin D1 in Lmna<sup>−/−</sup> cells did not rescue the defect in stalled forks or new origins (Fig. 8E and F). These results

FIG 7 Reinitiation of stalled DNA replication forks and initiation of new origins in Lmna<sup>+/+</sup> and Lmna<sup>−/−</sup> cells. (A) Shown are DNA labeling and HU treatment protocol for single DNA fiber analysis (i) and three major types of labeled DNA tracts for analysis (ii). (B) Comparison of global DNA replication restart after release from 2 h of HU treatment. Cells were prelabeled with IdU, treated with HU, and then postlabeled with CldU, fixed, immunostained with IdU (green) and CldU (red) antibodies, and counterstained with DAPI (blue). Equal intensities of CldU and IdU as well as strong colocalization was observed in Lmna<sup>+/+</sup> MEFs, indicating that DNA replication was able to restart. In contrast, CldU staining in Lmna<sup>−/−</sup> cells was very weak, and little colocalization was detected. (C) Quantification of percentages of cells with stalled forks after HU treatment. Lmna<sup>−/−</sup> cells have the least incorporation of CldU and thus the maximum frequency of cells with stalled forks. (D) Representative images of replication tracks from Lmna<sup>+/+</sup> and Lmna<sup>−/−</sup> cells after 1 h of HU treatment (i), after 21 h of HU treatment (ii), and in cyclin D1-depleted cells after 21 h of HU treatment (iii). (E) Quantification of stalled forks determined by fiber analysis with only IdU signal after 1 or 21 h of HU treatment. Lmna<sup>−/−</sup> cells ectopically expressing lamin A were designated Lmna<sup>−/−</sup>+ lamin A. (F) Quantification of new origins as determined by CldU signal after 1 or 21 h of HU treatment. (G) Distribution of IdU track length from DNA fibers from control Lmna<sup>+/+</sup> and Lmna<sup>−/−</sup> cells and Lmna<sup>−/−</sup> and Lmna<sup>−/−</sup> cells treated with HU for 5 h.
suggested that lamin A has a critical role in the process of stalled replication fork resolution.

Stalled replication fork collapse results in DNA double-strand breaks (DSBs) that are repaired by HR. Since lamin A/C-deficient cells have a high frequency of stalled replication forks, which do not collapse in order to initiate the process of HR for DNA repair, we examined whether DNA DSB repair by HR is different in cells with and without lamin A/C. Since cyclin D1 interacts with Rad51 and cyclin D1 is associated with lamin A (Fig. 1G), we expected defective HR repair of DNA DSBs in lamin A/C-deficient cells. However, the frequency of HR repair of a green fluorescent protein (GFP) reporter gene (pDR-GFP) in MCF7 cells after lamin A/C depletion with siRNA (Fig. 9A) was unaltered from the level in control-transfected cells (Fig. 9B), suggesting that lamin A/C does not have a role in DNA DSB repair via the HR pathway.

Lamin A/C is localized to the inner nuclear membrane, and telomeres are known to be associated with the nuclear matrix (44, 46). Telomeres of two sister chromatids at the end of a duplicated chromosome can recombine, and these exchanges are an indicator of relaxed control of DNA repair at telomeres. We visualized metaphase chromosomes by chromosome orientation fluorescent in situ hybridization (CO-FISH) (26, 36) and measured telomere region recombination events in Lmna−/− and Lmna+/+ cells (Fig. 9C). Neither Lmna−/− nor Lmna+/+ cells displayed any loss of telomere signals or telomere-specific sister chromatid exchanges, indicating that lamin A/C deficiency does not affect telomere stability or global sister chromatid exchange repair processes. Similarly, analysis of Lmna−/− and Lmna+/− cells for telomere circular DNA (35, 47) did not detect any difference in new arc telomeric DNA (Fig. 9D), indicating a normal recombination phenotype at the telomeres. Thus, the role of lamin A/C in DNA DSB repair is minimal, supporting the argument that lamin A/C plays a critical role in the restart of stalled replication forks (Fig. 9E).

**DISCUSSION**

LMNA mutations can result in synthesis of altered lamin proteins termed progeria. The accumulation of LMNA mutations may cause chromatin perturbations affecting DDR and DSB production (16). Furthermore, progerin accumulation is proposed to disrupt some replication and repair factor functions. As a result, xeroderma pigmentosum group A protein may mislocate to replication forks and affect replication fork stalling with subsequent DNA DSB repair (37). While lamin A/C-deficient cells have decreased cyclin D1 protein levels, they do not display any major IR response defects. Instead, lamin A/C is required for efficient repair of damage induced by agents which cause DNA adducts or lesions that are repaired mostly during S phase. In cultured cells, DNA replication sites can be visualized as discrete early or late replication foci in the nucleoplasm (48), and lamin A/C is present at sites of early replication in normal human fibroblasts (34, 49). The prevalence of triradial chromosomes after treatment with MMC, cisplatin, or HU, as well as the impaired recruitment of DNA polymerase η to UV550 laser-induced damage in Lmna−/− cells, strongly suggests defective resolution of stalled replication forks. Replication fork restart after deoxynucleotide pool depletion indicates how quickly cells are able to recover from a replication block and resume normal DNA synthesis. Lamin A/C-depleted cells were unable to restart most replication forks after treatment with HU compared to cells with lamin A/C, indicating that lamin A/C is required for the resolution of stalled replication forks. DNA fiber analysis confirmed this requirement and indicated that lamin A/C deficiency resulted in shorter track lengths. Since lamin

![Figure 8](http://mcb.asm.org/1219)
A/C-deficient cells have normal homology-directed repair as well as repair of IR-induced DNA damage, even in the absence of cyclin D1, such repair may be mechanistically different from ICL and stalled fork repair.

Based on these observations, we speculate that lamin A/C provides a platform for the resolution of stalled replication fork intermediates (Fig. 9E). The platform requires both lamin A/C and cyclin D1. Since cyclin D1 is released from lamin A after HU treat-
ment, release of cyclin D1 from lamin A may be regulating an activity, possibly a nucleolytic step, required for collapse of stalled replication forks prior to initiation of homology-directed repair. Moreover, cyclin D1 interacts with Rad51, which may assist repairosome formation. This is consistent with defective Rad51 focus formation, as well as other repairosome factors related to resection, after HU or cisplatin treatment in Lamina-/- cells or cells depleted of cyclin D1. These and other results suggest that the absence of lamin A/C and cyclin D1 impacts the resolution of the stalled replication fork. Based on our results, we propose that lamin A/C provides a platform (Fig. 9E) with a protective function during replication fork stalling that is dependent upon cyclin D1. We also provide evidence that the absence of lamin A/C and cyclin D1 impacts the resolution of the stalled replication fork. Based on our results, we propose that lamin A/C provides a platform (Fig. 9E) with a protective function during replication fork stalling that is dependent upon cyclin D1. We also provide evidence that the absence of lamin A/C and cyclin D1 impacts the resolution of the stalled replication fork.

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45. Reference deleted.
ERRATUM

Lamin A/C Depletion Enhances DNA Damage-Induced Stalled Replication Fork Arrest


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Volume 33, no. 6, p. 1210–1222, 2013. Page 1215: In Fig. 4B, “min” should be “hr.”

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