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Report

# Inner Nuclear Envelope Proteins SUN1 and SUN2 Play a Prominent Role in the DNA Damage Response

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## Summary

**The DNA damage response (DDR) and DNA repair are critical for maintaining genomic stability and evading many human diseases [1, 2]. Recent findings indicate that accumulation of SUN1, a nuclear envelope (NE) protein, is a significant pathogenic event in Emery-Dreifuss muscular dystrophy and Hutchinson-Gilford progeria syndrome, both caused by mutations in *LMNA* [3, 4]. However, roles of mammalian SUN proteins in mitotic cell division and genomic stability are unknown. Here we report that the inner NE proteins SUN1 and SUN2 may play a redundant role in DDR. Mouse embryonic fibroblasts from *Sun1*$^{-/-}$*Sun2*$^{-/-}$ mice displayed premature proliferation arrest in S phase of cell cycle, increased apoptosis and DNA damage, and decreased perinuclear heterochromatin, indicating genome instability. Furthermore, activation of ATM and H2A.X, early events in DDR, were impaired in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ fibroblasts. A biochemical screen identified interactions between SUN1 and SUN2 and DNA-dependent protein kinase (DNAPK) complex that functions in DNA nonhomologous end joining repair and possibly in DDR [2, 5, 6]. Knockdown of DNAPK reduced ATM activation in NIH 3T3 cells, consistent with a potential role of SUN1- and SUN2-DNAPK interaction during DDR. SUN1 and SUN2 could affect DDR by localizing certain nuclear factors to the NE or by mediating communication between nuclear and cytoplasmic events.**

## Results

### *Sun1*$^{-/-}$*Sun2*$^{-/-}$ Mouse Embryonic Fibroblasts Exhibited Premature Proliferative Arrest at the S Phase of the Cell Cycle

SUN proteins are inner nuclear membrane proteins with their N-terminal region localized in the nucleoplasm and their C-terminal SUN domain in the lumen of the nuclear envelope (NE) [7–9]. We have previously used mouse genetics to analyze the physiological functions of SUN1 and SUN2 and found that *Sun1*$^{-/-}$*Sun2*$^{-/-}$ mice died shortly after birth [10, 11]. Although the neonatal death phenotype was partly rescued by expressing SUN1 in the nervous system, the surviving mice still displayed multiple defects including growth retardation [10, 12], prompting us to examine the function of SUN1 and SUN2 in mitotic cell division and genomic stability in mouse embryonic fibroblasts (MEFs).

The MEFs were isolated from embryos at embryonic day 14.5 (E14.5). MEFs from the *Sun1*$^{-/-}$*Sun2*$^{-/-}$ mice, but not *Sun1*$^{-/-}$ or *Sun2*$^{-/-}$ mice, proliferated significantly more slowly than wild-type (WT) MEFs after passage 5 (Figure 1A; see Figure S1 available online). Cell-cycle analysis on unsynchronized cells from passage 6 via flow cytometry showed that the G0/G1-phase fraction was only slightly increased and the S phase fraction was slightly reduced in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs (Figure 1B). In contrast, using bromodeoxyuridine (BrdU) to label the replicative DNA in S phase cells, we observed that the percentage of proliferative S phase cells in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs was less than half that of WT MEFs (Figure 1C), suggesting an S phase arrest in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs. Furthermore, there were an increased number of annexin V-positive cells in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs at passage 6 (Figure 1D), indicating an increase in apoptosis. These results raised the possibility that DNA damage accumulated more rapidly in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs.

### *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs Exhibit Excessive DNA Damage

To detect the potential genomic instability in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs, we carried out single-cell electrophoresis to observe the level of DNA damage. In the absence of methyl methanesulfonate (MMS), which induces DNA damage [13], there was no significant difference in the tail moment between WT and *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs. After treatment with MMS, we observed a significant increase in the number of *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs with prominent comet tails, indicative of DNA fragmentation (Figure 1E). In addition, using transmission electronic microscopy (TEM), we found that the perinuclear heterochromatin was decreased in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs (Figure 1F). These results suggested that SUN1 and SUN2 have roles in maintaining genomic stability, possibly by affecting DDR and/or DNA repair.

### DDR Was Impaired in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs

Phosphorylation of ataxia telangiectasia mutated protein (ATM) and H2A.X, a histone H2A variant, at Ser139 (i.e., $\gamma$-H2A.X) are among the earliest events to occur in response to DNA damage [2, 14, 15]. These early DDR events lead to activation of DNA repair factors and cell-cycle checkpoints, ensuring the proper repair of sites of DNA damage [2, 15, 16]. We obtained three pieces of data to indicate that the early events in DDR are affected in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs. First, the expression level of $\gamma$-H2A.X was significantly reduced in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs (Figure 2A). In addition, the level of phosphorylated Chk1, a cell-cycle checkpoint factor downstream of the DDR pathway, was also reduced (Figure 2A). Second, although ATM was seen to be activated by 0.1 $\mu$M of hydroxyurea (HU) in WT MEFs, it was not activated by HU in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs (Figure 2B). Third, we found that

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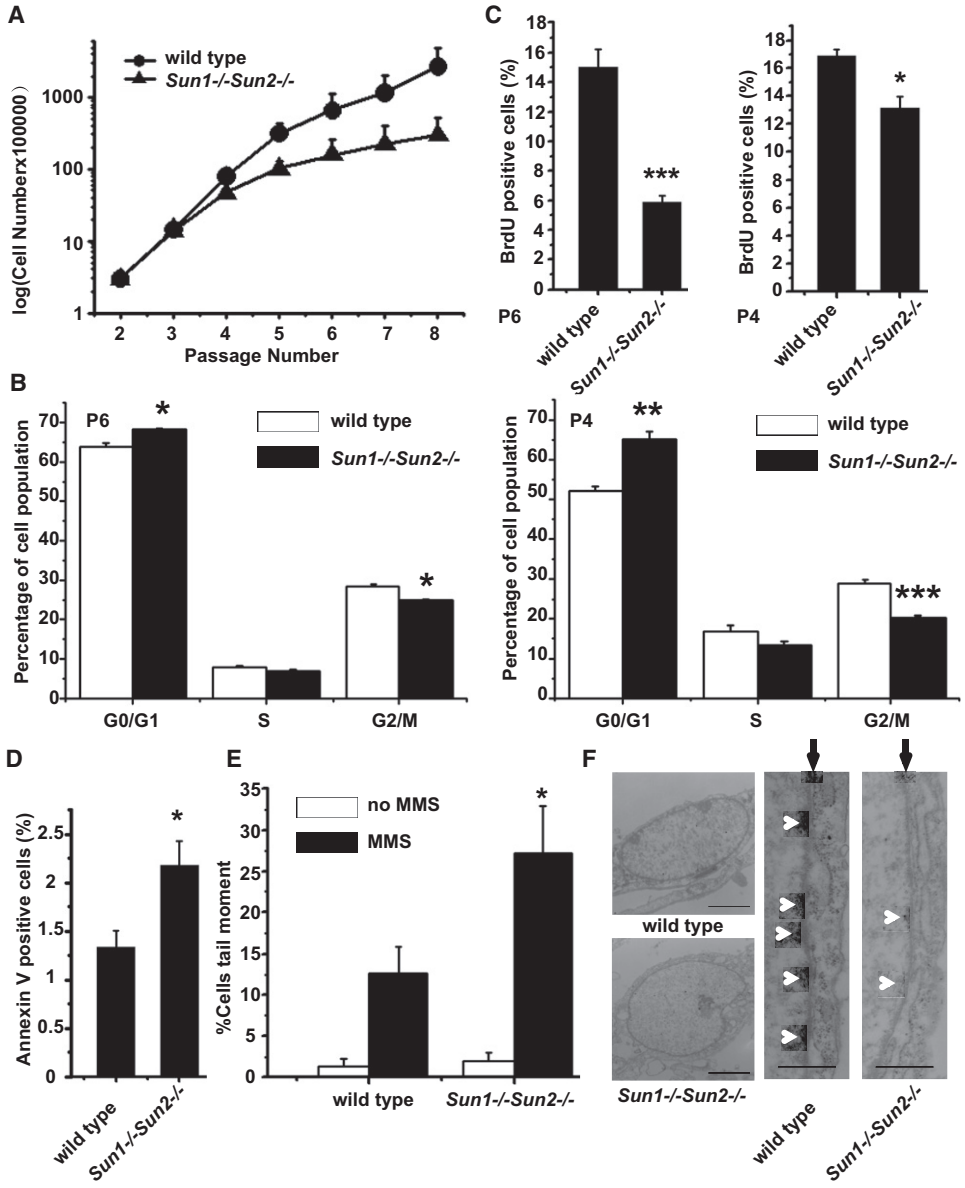


Figure 1. *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs Exhibit Premature Proliferative Arrest and Genomic Instability

(A) Growth curve of MEFs of the indicated genotypes from passage (P)2 to P8. The results were from three independent cell lines of *Sun1*$^{-/-}$*Sun2*$^{-/-}$ and WT controls.

(B) Histogram showing the cell-cycle distribution of MEFs at P4 and P6. Only small changes in the cell population at G0/G1 and G2/M were observed. *$p < 0.05$.

(C) Histogram showing the percentage of BrdU-positive MEFs at P4 and P6. S phase cell arrest is indicated by the dramatic decrease in BrdU-positive cells in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs. Data were calculated from three replicates of two cell lines for each genotype. ***$p < 0.001$.

(D) Histogram showing the percentage of Annexin V-positive MEFs undergoing apoptosis, indicating a significant increase in apoptosis in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ cells at P6. Data were calculated from three replicates of two cell lines for each genotype. *$p < 0.05$.

(E) Histogram showing a significant increase in the percentage of the tail moment in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs at P5 after MMS treatment, indicating an increase in DNA damage. Data was analyzed using the Comet Score software (TriTek). n = 54. *$p < 0.05$.

(F) Representative TEM images showing the structure of the NE (arrows) and a significant decrease in perinuclear heterochromatin (arrowheads) in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs at P5. Scale bars for whole nucleus images represent 5 μm. Scale bars for enlarged images represent 500 nm.

All error bars in each graph represent SEM. See also Figure S1.

the cell division cycle of *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs was not blocked at the G2/M phase following treatment with 200 ng/μl of mitomycin C (MMC) (Figure 2C), indicating that the mutant cells failed to properly respond to DNA damage. However, due to the lack of a suitable antibody for mouse nonphosphorylated ATM, we could not exclude the possibility that the observed decrease of phosphorylated ATM was partly due to apoptosis-induced ATM degradation [17]. However, such an effect of apoptosis is unlikely to be significant because our analysis using annexin V indicated that apoptosis was not dramatically increased in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs (only 2.2%, compared to 1.3% in WT; Figure 1D). To confirm the defect

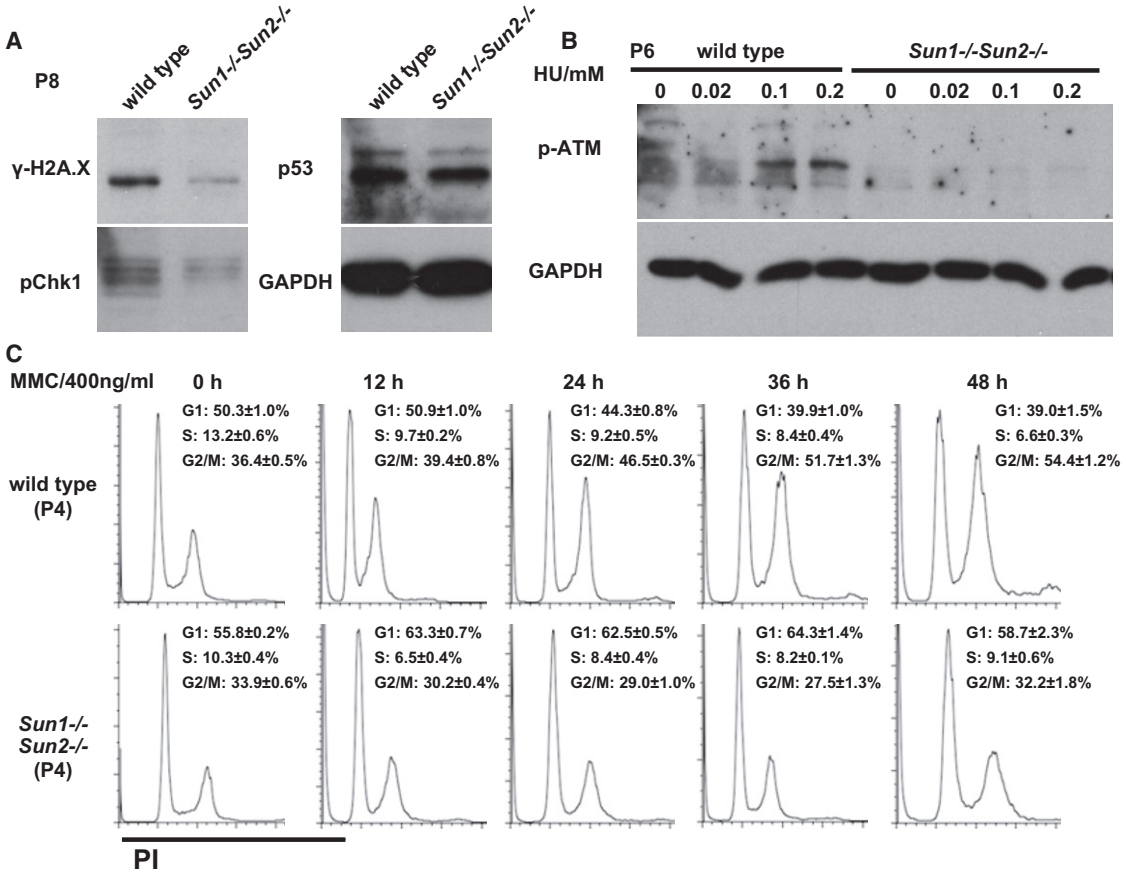


Figure 2. *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs Exhibit Defects in DDR

(A) Results of immunoblot analysis showing that the protein levels of γ-H2AX and phosphorylated Chk1 (pChk1), but not that of p53, are significantly reduced in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs at P8. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control.

(B) Result of immunoblot analysis showing that activation (phosphorylation) of ATM (pATM) following HU treatment was significantly reduced in *Sun1*$^{-/-}$ *Sun2*$^{-/-}$ MEFs at P6. GAPDH was used as the internal control.

(C) Representative cell-cycle profiles of PI-stained MEFs after MMC treatment at P4. MMC failed to block *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs at the G2 phase, indicating that DDR was impaired in the mutant cells.

See also Figure S2.

of *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs in DDR, we examined the sensitivity of *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs to various DNA-damaging agents. Although *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs exhibited no significant abnormality in their response to γ-irradiation, they exhibited increased sensitivity to MMS and MMC (Figure S2). These results suggest that SUN1 and SUN2 have a prominent role in DDR to specific types of DNA damage.

### SUN1 and SUN2 Interact with the DNAPK Holoenzyme

To search for the mechanism of SUN1 and SUN2 function in DDR, we screened for SUN1 interacting proteins by applying tandem affinity purification and MALDI-MS/MS proteomic analysis [18]. The effectiveness of this approach was indicated by the identification of, among only 27 candidate proteins (Table S1), three KASH domain proteins (Syne-1/Nesprin-1, Syne-2/Nesprin-2, and Nesprin-3) that have all been well characterized as biochemical and functional partners of SUN1 and SUN2 [10, 11, 19–21]. In addition, several cytoskeleton proteins and emerin were also identified (Table S1), and this was likely due to their interactions with the Syne/Nesprin proteins [22].

DNAPKcs, the catalytic subunit of the DNAPK holoenzyme, which also includes Ku70 and Ku80 as the regulatory subunits [23], was one of candidate SUN1-associated proteins. The DNAPK holoenzyme has been studied extensively for its role in the nonhomologous end-joining repair pathway [2, 5, 24]. Recently, DNAPKcs was found to interact with the Hutchinson-Gilford progeria syndrome (HGPS) mutant version of Lamin A/C, linking its function to HGPS-related DNA instability and cell aging [25]. Although DNAPKcs has been reported to have a role in the phosphorylation of H2A.X in experiments using DNAPKcs$^{-/-}$ MEFs [6], its function in the early steps of DDR and the potential mechanism of such a role are not clear.

Based on coimmunoprecipitation (coIP) and western blot analysis, we confirmed that DNAPKcs was associated with both SUN1 and SUN2 (Figures 3A and 3B). Similar experiments showed that both Ku70 and Ku80 also interacted with SUN1 and SUN2 (Figures 3C–3F). We further examined the localization of these proteins by immunofluorescence staining of tagged proteins expressed from transformed plasmids. Consistent with previous studies, DNAPKcs, Ku70, and Ku80 were localized uniformly in the nucleus (Figure 4; Figures S4A–S4G [25, 26]). Dual-staining analysis with SUN1 and SUN2 and these components of the DNAPK complex indicated that these proteins colocalized at a low level along the inner side of the NE (Figure 4A; Figures S4A–S4G). However, we

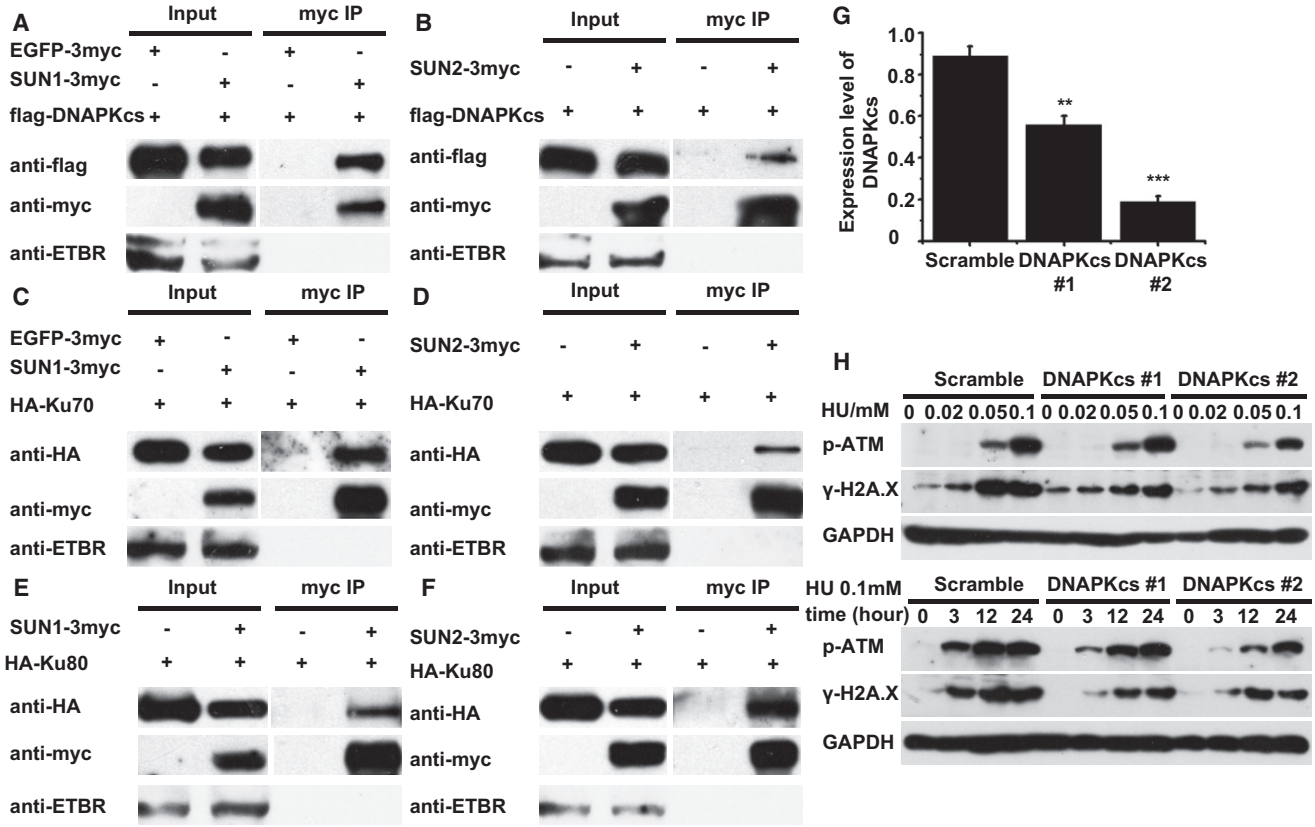


Figure 3. SUN1 and SUN2 Interact with DNAPKcs, Ku70, and Ku80

(A–F) Lysates from 293T cells expressing combinations of GFP-myc, flag-DNAPKcs, HA-Ku70, HA-Ku80, SUN1-3myc, or SUN2-3myc were immunoprecipitated with an anti-c-*myc* antibody and examined by immunoblot analysis. Interactions were detected between either SUN1 or SUN2 and each subunit of the DNAPK complex (DNAPKcs, Ku70, and Ku80) using antibodies as indicated. ETBR was used to exclude the possibility that DNAPK complex was immunoprecipitated with DNA.

(G) Histograph showing the relative level of the DNAPKcs mRNA in NIH 3T3 cells infected with one of three lentivirus strains, which each expressed a unique shRNA. shRNA #2, targeting a sequence within exon 43 of DNAPKcs, was much more effective at knocking down the mRNA level than shRNA #1, which targeted a sequence within exon 10 of the gene. Error bars represent SEM.

(H) Results of immunoblot analysis showing the effect of DNAPKcs knockdown on phosphorylation of ATM at residue S1841 after HU treatment using an antibody against pATM. Cells infected with shRNA #2 displayed a significant reduction in ATM activation when phosphorylation was assayed either by varying the concentration of HU (upper panel) or by changing the time after the HU treatment (lower panel).

See also Figure S3.

did not observe an increase in this colocalization after HU treatment (Figure 4B). We further compared the localization of endogenous Ku70 in WT and *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs but also did not observe a significant difference under standard culturing conditions with or without the HU treatment (Figures S4H–S4K). Because we cannot make a conclusion about the function of the interaction between SUN1 and SUN2 and DNAPK complex, and their colocalization in DDR, the mechanism by which the DNAPK complex interacts with SUN1 and SUN2 remains to be understood.

Given the well-known function of the DNAPK complex in DNA repair, the interaction between SUN1 and SUN2 and the DNAPK complex may suggest that SUN1 and SUN2 have a function downstream of DDR, especially in DNA repair, which is consistent with the suggestion that Lamin A/C has a role in DNA repair. However, the data presented earlier indicated a role for SUN1 and SUN2 in an early step of DDR. Using small hairpin RNA (shRNA) to knockdown the DNAPKcs messenger RNA (mRNA) level in NIH 3T3 cells (Figure 3G), we observed a reduction of ATM and H2A.X phosphorylation when the cells were treated with HU (Figure 3H), suggesting that the interaction between SUN1 and SUN2 and the DNAPK complex is potentially involved in mediating the role of SUN1 and SUN2 in DDR. However, we cannot exclude the possibility that the reduction of ATM and H2AX phosphorylation in this experiment is solely caused by knocking down DNAPKcs. Due to the lack of an appropriate antibody against the mouse phosphorylated DNAPKcs, we could not examine whether SUN1 and SUN2 play a role in activating the DNAPKcs in DDR.

## Discussion

The mammalian SUN1 and SUN2 proteins have been studied for their roles in nuclear migration and anchorage as well as in anchoring meiotic telomeres to the NE during animal development [10–12, 27, 28]. In this study, we showed that these two inner NE proteins also have a significant function in DDR. Like their roles in anchoring myonuclei and neuronal migration, SUN1 and SUN2 functions in DDR are likely redundant; only MEFs from double knockout mice display obvious defects. We can speculate on a potential model for their function based on our limited observations and the available information.

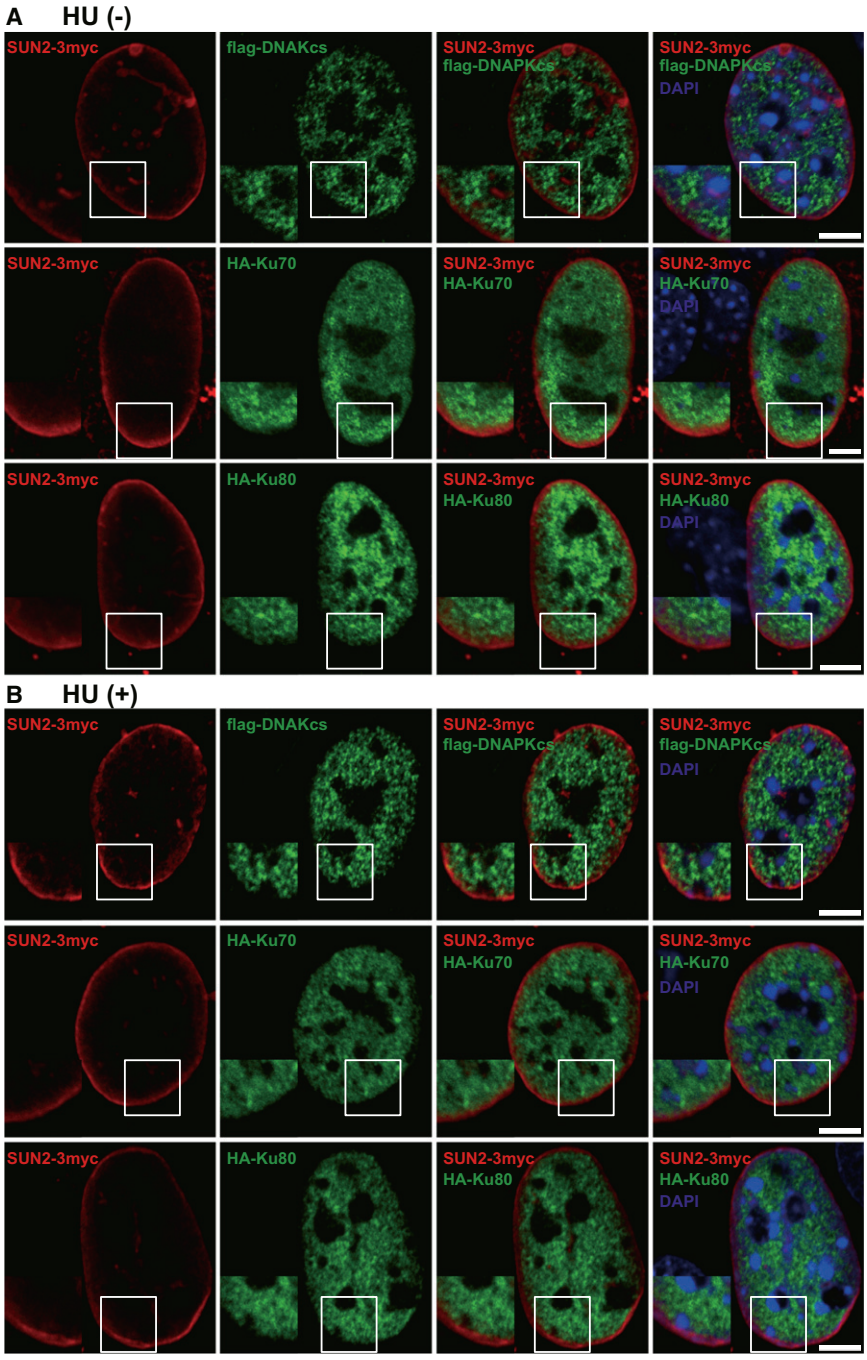


Figure 4. The Colocalization of SUN2 and the DNAPK Complex Is Not Increased after HU Treatment

(A). Fluorescence images showing localization of tagged proteins (as indicated) in NIH 3T3 cells without HU treatment.

(B) Fluorescence images showing localization of tagged proteins (as indicated) in NIH 3T3 cells after HU treatment for 24 hr. The four panels in each line show the staining of SUN2, one of the three DNAPK complex subunits, and two merged images. The NE-spanning SUN2 proteins appear to colocalize at a low level with the nucleoplasmic-distributed DNAPK complex along the inner side of the NE. Scale bars represent 5 μm. See also Figure S4.

The identification of the interaction between SUN1 and SUN2 and the DNAPK complex provides an important mechanistic clue. Because DNAPK is better known for its function in DNA repair, we can consider two different hypotheses regarding the function of this interaction. One hypothesis is that SUN1 and SUN2 may interact with DNAPK for their function in DNA repair, and a defect in this function was masked by the defect in the earlier DDR events in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs. SUN1 and SUN2 function in DDR would thus be mediated by factors that are yet to be determined. An alternative hypothesis is that the DNAPK complex also has a significant role in DDR and its interaction with SUN1 and SUN2 is critical for such a function. This hypothesis is consistent with a previous report that DNAPKcs has a role in H2A.X phosphorylation [6] and our result that shRNA knockdown of DNAPKcs compromised ATM and H2A.X activation in NIH 3T3 cells (Figure 3H). It is conceivable that SUN1 and SUN2 function in DDR by localizing DNA damage sites or certain DDR factors to the NE. In a yeast study, Ku70, a regulatory subunit of DNAPK, was shown to recognize and recruit the site of DNA damage to the NE in an Mps3-dependent manner [29], but it is not clear whether this NE localization is for DDR or DNA repair. In this study, we observed the colocalization between DNAPK components and SUN1 and SUN2 in mammalian cells (Figure 4; Figures S4A–S4G), indicating a similar function to their yeast counterparts. However, we did not observe an increase of this colocalization after HU treatment (Figure 4; Figures S4H–S4K). In addition, the localization of endogenous Ku70 was not changed in *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs (Figures S4H–S4K). Therefore, it is possible that the SUN1 and SUN2 interaction with the DNAPK complex is a constitutive cellular event required for proper DDR, and the interaction is not required for just the NE localization of DNAPK.

SUN proteins are known to form the NE complex with outer NE KASH-domain proteins that interact with cytoplasmic factors [10–12, 19, 20, 22]. Therefore, an alternative model for the roles of SUN1 and SUN2 in DDR could be that they mediate the communication between nuclear and cytoplasmic events. In our search for SUN1 and SUN2 interacting factors, we also identified the $Ca^{2+}$-binding protein reticulocalcin-2 (Rcn2), which has been suggested to be localized in the lumen of the endoplasmic reticulum (ER) [30] and has been shown to have a role in activating ERK1 and ERK2 in a recent report [31]. Interestingly, *Sun1*$^{-/-}$*Sun2*$^{-/-}$ MEFs displayed impaired ERK activation after HU and MMC treatment (Figures S3E–S3F). Our analysis using coIP and immunostaining confirmed the interaction between SUN1 and SUN2 and Rcn2 and indicated that they colocalized on to the NE (Figures S3G–S3K).

However, when NIH 3T3 cells were treated with shRNA against *Rcn2*, we failed to identify any effect on ATM activation or subsequent after the treatment to induce DDR induction (Figures S3L–S3N). Though this negative result is not sufficient to exclude a role of Rcn2 in DDR due to potential redundant functions, the physiological role of the interaction between SUN1 and SUN2 and Rcn2 is currently unclear.

Lamin A/C are part of the nuclear lamina located inside the nuclear inner membrane, and their functions have been linked to many important cellular events [32–34]. Both SUN1 and SUN2 have been shown to interact with Lamin A/C [20, 35, 36], and the HGPS-associated Lamin A/C mutations have been shown to impair the interaction between Lamin A/C and SUN1 and SUN2 [37]. These data raise a possibility that SUN1 and SUN2 may function in DDR through this interaction with Lamin A/C. However, several studies on Lamin A/C contradict such a model. For example, the HGPS mutant version of Lamin A/C (termed "progerin"), but not WT, was found to interact with DNAPKcs in a recent study [25], even though the mutant Lamin A/C cannot bind to SUN1 and SUN2 [37]. Furthermore, unlike *Sun1*$^{-/-}$*Sun2*$^{-/-}$, Lamin A/C mutations were found to cause an increase in $\gamma$−H2A.X levels, which were attributed to defective DNA repair [38, 39]. Therefore, the role of the SUN1 and SUN2 interaction with Lamin A/C in DDR is still unclear. Chen et al. recently reported that accumulation of SUN1 is a pathogenic event in Emerry-Dreifuss muscular dystrophy and Hutchinson-Gilford progeria, which are caused by mutations in *LMNA* [3]. Eliminating or reducing SUN1 was found to significantly relieve some pathological phenotypes characterized in mouse models of these diseases. Our results may provide valuable insight into the potential mechanism underlying these observations. We show that SUN1 and SUN2 act redundantly to promote DDR, whereas LMNA mutations were shown to cause potential increases in DDR [39]. Therefore, it is logical to propose that some of the disease phenotypes are caused by hyperactivity in DDR as the result of an abnormally high level of SUN1. Mutating *Sun1* is expected to only reduce the level of DDR, but the reduction may be sufficient to neutralize the effect of the *LMNA* mutations. Further studies are needed to uncover the molecular mechanism by which SUN1 and SUN2 affect DDR.

## Experimental Procedures

All animal-related procedures were reviewed and approved by the Institute of Developmental Biology and Molecular Medicine Institutional Animal Care and Use Committee.

### Cell Culture and Proliferation Assay

We prepared MEFs from E14.5 embryos and cultured them in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (Invitrogen). For the continuous passage assay, we plated MEFs at a density of $3 \times 10^5$ cells in a 6 cm plate. We then counted and replated the cell every 3 days. The BrdU incorporation assay was carried out according to a standard protocol [40]. Briefly, $5 \times 10^4$ MEFs were plated in each well of a 6-well plate. After incubation for 24 hr, they were treated with 10 mg/ml BrdU (Sigma) for 4 hr. The cells were then harvested and stained with a fluorescein isothiocyanate-conjugated anti-BrdU antibody (Caltag) and propidium iodide (PI) (Sigma) or 7-amino-actinomycin D. The cell-cycle distribution was analyzed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest (BD Biosciences) and FlowJo (Tree Star) software.

### Statistic Methods

Data were calculated using an unpaired two-tailed Student t test and presented as means ± SEM.

### Supplemental Information

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.06.043.

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