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Roles of POLD4, smallest subunit of DNA polymerase δ, in nuclear structures and genomic stability of human cells

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ABSTRACT

Mammalian DNA polymerase δ (pol δ) is essential for DNA replication, though the functions of this smallest subunit of POLD4 have been elusive. We investigated pol δ activities *in vitro* and found that it was less active in the absence of POLD4, irrespective of the presence of the accessory protein PCNA. shRNA-mediated reduction of POLD4 resulted in a marked decrease in colony formation activity by Calu6, ACC-LC-319, and PC-10 cells. We also found that POLD4 reduction was associated with an increased population of karyomere-like cells, which may be an indication of DNA replication stress and/or DNA damage. The karyomere-like cells retained an ability to progress through the cell cycle, suggesting that POLD4 reduction induces modest genomic instability, while allowing cells to grow until DNA damage reaches an intolerant level. Our results indicate that POLD4 is required for the *in vitro* pol δ activity, and that it functions in cell proliferation and maintenance of genomic stability of human cells.

Introduction

Eukaryotic DNA polymerase δ (pol δ), a key enzyme that participates in DNA replication and repair, consists of four subunits; POLD1 (catalytic subunit, alternatively called p125), POLD2 (p50), POLD3 (p68), and POLD4 (p12) [1,2]. Among those, POLD4 binds to POLD1, POLD2, and an accessory protein of PCNA, which allows pol δ to exhibit its full activity [1,3].

A previous study showed that the *POLD4* ortholog of *Cdm1* in *Schizosaccharomyces pombe* is a non-essential gene related to cell growth, division, and sensitivity to DNA damaging reagents [4]. *Saccharomyces cerevisiae* does not have a POLD4 counterpart, indicating that POLD4 is dispensable in lower eukaryotic cells. In contrast, siRNA-mediated knockdown of POLD4 caused a significant decrease in the proliferation rate of FGF2-activated mouse-endothelial cells [5]. However, it remains unknown whether POLD4 is required for other types of mammalian cells, such as those related to human cancer, or if it has additional functions in mammalian cells.

In the present study, we analyzed the roles of POLD4 for cell proliferation in human lung cancer cell lines. Our findings indicate that POLD4 is required for maintaining the proper nuclear structures and suggest that the pathological structures reflect elevated DNA damage in chromosomes.

Materials and methods

Antibodies. The antibodies used in this study were anti-POLD4 (POLD4 subunit of pol δ) ascites (2B11, Abnova, Taipei City, Taiwan), anti-lamin B (c-20) (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-γ-tubulin (Sigma–Aldrich, St. Louis, MO).

In vitro pol δ activity. Three- and 4-subunit DNA from pol δ were expressed in *Escherichia coli*, and purified as described previously [6]. pol δ activity was determined in a reaction mixture (25 μl) containing 20 mM HEPES–NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM each of dGTP, dATP, dCTP, and [α-^{32}P]dTTP, 100 ng poly dA-oligo dT (GE Healthcare, Piscataway, NJ), 86 ng (1.0 pmol as a trimer) of PCNA, and 11–88 ng (46–372 fmol) of pol δ at 30 °C for 10 min. Following incubation, the reactions were terminated with 2 μl of 300 mM EDTA. pol activity was determined with reference to the incorporation of [α-^{32}P]dTMP, as previously described [6].

Colony formation assay. To assess cell proliferation, colony formation assays were performed as previously described [7]. In order to rule out the off-target effect, we designed two independent DNA sequences as follows: MS543F, 5′-GATCCCCagtctctggcatctctatcATTCAAGAGATgatagagatgccagagactTTTTTGGAAA-3; MS544R, 5′-AGCTTTTCCAAAAAagtctctggcatctctatcATCTCTTGAATgatagagatgccagagactGGG-3; MS551F, 5′-GATCCCCgcatctctatcccctatgaATTCAAGAGATtcataggggatagagatgcTTTTTGGAAA-3; and MS552R, 5′-AGCTTTTCCAAAAAgcatctctatcccctatgaATCTCTTGAATtcataggggatagagatgcGGG-3, in which the targeting sequences are indicated in lowercase letters. To construct shRNA vectors, MS543F and MS544R

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(polD4-5), and MS551F and MS552R (polD4-3-1) were annealed, then inserted between the restriction sites BglII and HindIII in PH1RNAneo [7]. Cells transfected with a vector carrying either polD4-3-1 or polD4-5 were cultured in media containing 1 mg/ml G418 (Invitrogen, Carlsbad, CA 10131-027), which was reduced by 0.2 mg/ml every 2 days until it reached 0.4 mg/ml. When colonies grew to visible seizes, they were fixed by cold methanol for 5 min and stained with 4% Giemsa for 15 min at room temperature.

RNA interference. Transfection was carried out using 50 nmol/L of a small interfering RNA (siRNA) duplex (Sigma–Aldrich) targeting each mRNA, or negative control 1# (Ambion) with Lipofectamine-2000 (Invitrogen). The sequences of si*POLD4* were the same as those of polD4-3-1: *POLD4* (si*D4*) sense, 5′-GCAUCUCUAUCCCCUAUGATT-3′; and antisense, 5′-UCAUAGGGGAUAGAGAUGCTT.

Laser scanning cytometry (LSC). Following an overnight culture, 3×10^5/ml Calu6 cells on coverslips were fixed by cold methanol, washed with PBS, and incubated with 1 mg/ml RNase A in 50 mM Tris–HCl, pH 7.5, at 37 °C for 1 h. Cells were further treated with 50 μg/ml propidium iodide in a mixture containing 180 mM Tris–HCl, pH 7.5, 180 mM NaCl, and 70 mM $MgCl_2$ for 15 min. Nuclei structures and DNA contents were analyzed using a Laser Scanning Cytometer (LSC, Olympus, Tokyo, Japan), with DNA content at the G1 peak regarded as 2N, though Calu6 cells carry a greater amount of DNA chromatin than normal cells.

Cell cycle synchronization. Calu6 cells were synchronized according to the method of Nakagawa et al. [8], with minor modifications. In brief, 24-h treatment with 2 mM thymidine was used to arrest exponentially proliferating cells in the S phase. Cells were then released from arrest by three washes in PBS and grown in fresh medium for 15 h, then 1 μM of aphidicolin was used for the second block for 10 h. After releasing by three washes in PBS, cells were incubated in RPMI1640 containing 5% fetal bovine serum and harvested at various time points.

Immunofluorescence. Following an overnight culture, 3×10^5/ml Calu6 cells on coverslips were transfected with siRNA as described above. After 48 h, they were fixed in 4% paraformaldehyde for 10 min at room temperature, followed by treatment with cold methanol for 2 min. The coverslips were washed three times in PBS, treated with PBS containing 0.25% Triton X-100 on ice for 30 min, and incubated with anti-lamin B or anti-γ-tubulin antibody overnight at 4 °C. The cells were then washed three times in PBS, incubated for 1 h with Alexa 488-conjugated secondary antibody (Molecular Probes, OR, USA), and analyzed using an Olympus BX60 (Olympus).

Results and discussion

DNA synthesis activities of pol δ with or without POLD4 in vitro

In order to analyze POLD4 functions related to intrinsic pol δ activity, 3- and 4-subunit structures of pol δ were expressed and purified. In the absence of POLD4, pol δ was less active than the holoenzyme in a reaction containing poly dA–dT as a template primer (Fig. 1A), with a similar result obtained when the accessory protein of PCNA was omitted from the reaction (Fig. 1B). These results are consistent with those of previous studies [1,3] and indicate that POLD4 is required for pol δ to exhibit its full catalytic activity.

POLD4 required for cell proliferation

A previous genetic study of *S. pombe* demonstrated that the *POLD4* ortholog of *Cdm1* is a non-essential gene for cell growth,

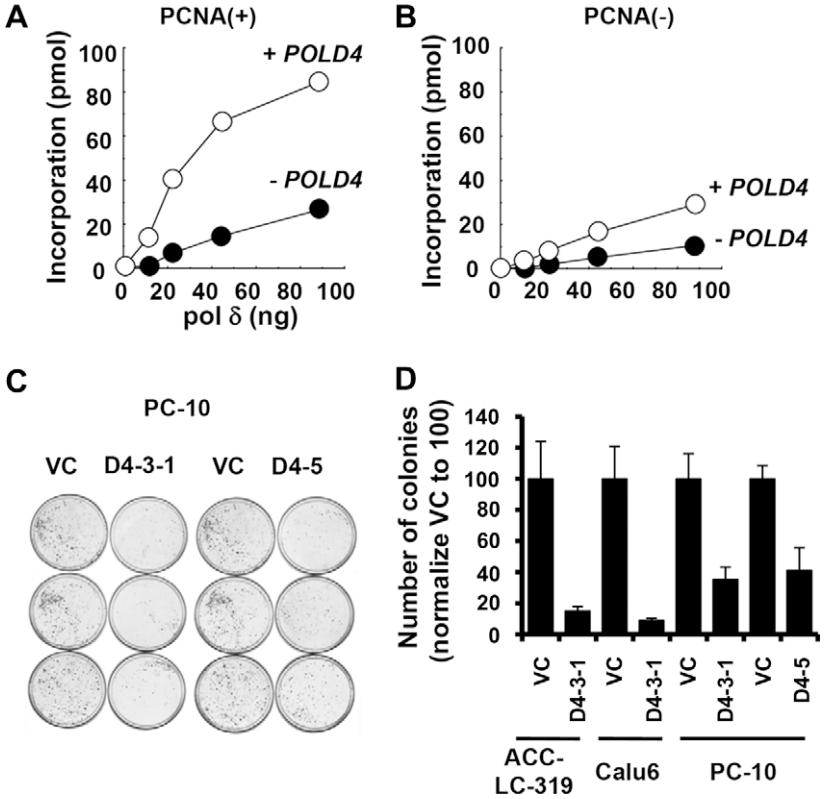

Fig. 1. *In vitro* DNA synthesis activities of pol δ and effect of POLD4 depletion on colony formation activity. (A) pol δ activities were measured and plotted as described in Materials and methods. (B) The same reactions were carried out in the absence of PCNA. (C) PC-10 was used for transfection with plasmids carrying either D4-3-1 or D4-5, and colony formation activity was determined as described in Materials and methods. VC represents vector control. (D) Results of the colony formation assay were plotted in a graph.

division, and sensitivity to DNA damaging reagents [4]. Nevertheless, it is possible that mammalian cells with larger genomic sizes require POLD4 for efficient and accurate DNA replication. We investigated this possibility using shRNA-mediated knockdown of *POLD4*. As shown in Fig. 1C, two independent sequences of shRNA caused reduced activity in a colony formation assay using PC-10, a human non-small cell lung cancer (NSCLC) cell line. Similar results were obtained with different NSCLC cell lines, Calu6 and ACC-LC-319 (Fig. 1D). These findings suggest that human cells require POLD4 for proliferation.

Structure and population of karyomere-like cells following siPOLD4 treatment

Since pol δ is a major DNA replication and repair polymerase, impairment of its activity may cause DNA replication stress, such as accumulation of single-stranded DNA gaps, and inefficient repair of endogenous DNA damage, which ultimately results in cell death. On the other hand, it is also possible that some cells continue to grow following such genetic erosion, which may cause genomic instability. Therefore, we investigated whether POLD4 is also required for suppressing genomic instability in human cells. Initially, we attempted to establish stable clones with low POLD4 expression using shRNA-treated cells. However, clones with adequate levels of POLD4 expression were gradually selected, leading to recovery to the original level over time (data not shown). Therefore, in the following experiments, we used siRNA to transiently reduce POLD4 expression (Fig. 2A, left).

Calu6 cells treated with si*POLD4* formed multiple or lobed nuclei, at a 5.3-fold greater frequency than in the control experiment (Fig. 2B and C). Similar structures were also observed when A549 cells were treated with si*POLD4* (data not shown). Staining with

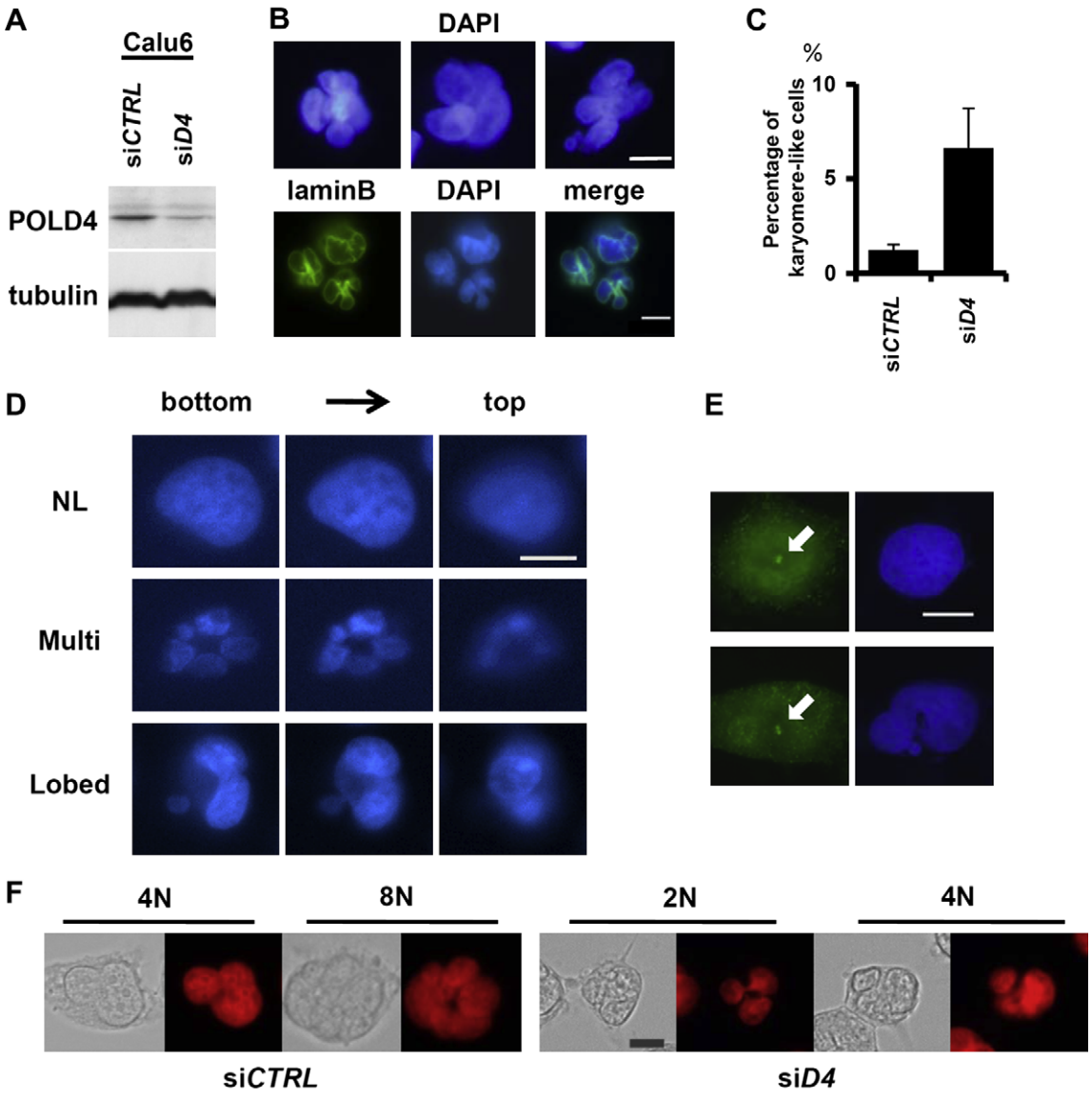

Fig. 2. Structures and population of karyomere-like cells upon si*POLD4* treatment. (A) Western blot analysis of POLD4 and α-tubulin in protein extracts from si*POLD4*- or si*CTRL*-treated Calu6 cells. (B) Upper panels: Calu6 cells were treated with si*POLD4* for 48 h and stained with DAPI. Sample karyomere-like nuclei are shown. Lower panels: Calu6 cells were treated with si*POLD4* for 48 h, then visualized with anti-lamin B antibody or DAPI. (C) Calu6 cells were treated with si*POLD4* or si*CTRL*, then the frequencies of karyomere-like structures in 1000 cells were counted and plotted. In this experiment, cells with three or more nuclear lobes, or three or more nuclei were regarded as karyomere-like cells. Averages of three independent results are shown with SD. (D) si*POLD4*-treated cells were stained by DAPI, then three sequential photographs were taken every 4 μm from the bottom. Upper, middle, and lower panels show images of normal, multiple, and lobed nuclear structures, respectively. (E) After being treated with si*POLD4*, cells were visualized with anti-γ-tubulin (left) or DAPI (right). Upper and lower panels show representative pictures of normal and karyomere-like nuclei, respectively. Centrosomes are indicated by arrows. (F) LSC analysis. Phase-contrast and propidium iodide-stained images of karyomere-like cells among 4N and 8N (si*CTRL*), or 2N and 4N (si*POLD4*) cells. Bar indicates 10 μm.

the anti-lamin B antibody outlined the edges of the DAPI structures and showed that the nuclear envelope was formed around chromatin (Fig. 2B, lower). Sequential acquisition of images from the bottom of the cells further illustrated the abnormal structures of single cells, including a flat profile and multiple nuclei (Fig. 2D, middle panels), or a single nucleus associated with multiple lobes (Fig. 2D, lower panels). For both types of abnormal structures, the nuclear sizes were approximately that of a normally shaped nucleus (Fig. 2D, upper panels).

The multiple nuclei seen with these structures were reminiscent of 'micronuclei' that indicated the presence of DNA damage and DNA replication stress in previous studies [9–11], while the lobed nuclei closely resembled 'karyomere' nuclei observed in zebrafish blastomeres [12] and early *Xenopus laevis* development [13]. In that latter study and other studies referenced therein, it was suggested that karyomere formation is a physiological mitotic process that may share similar mechanisms with pathological micronuclei formation; with both multiple and lobed nuclei, isolated chromosomes might

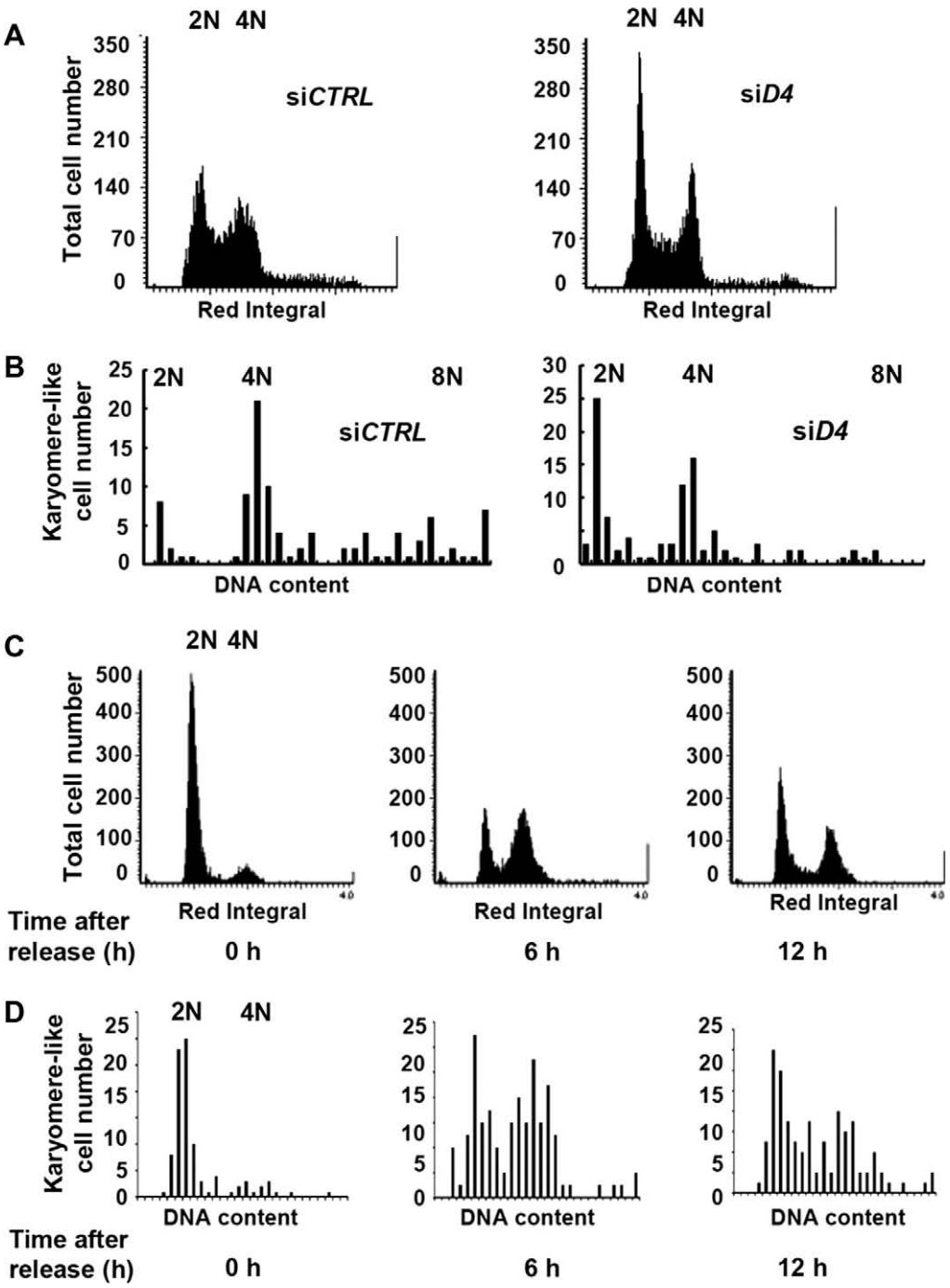

Fig. 3. Cell cycle dynamics of karyomere-like cells. (A) Calu6 cells were treated with si*CTRL* (left) or si*POLD4* (right), then their DNA contents were subjected to LSC analysis. The G1 population found among the majority of cells was regarded as 2N. (B) In the same experiment, DNA contents of 100 karyomere-like cells were determined. Cell numbers in each DNA content range were plotted with histograms. (C) Calu6 cells were treated with si*POLD4*, then synchronized at the G1/S boundary and released for cell cycle progression. At 0, 6, or 12 h after release, DNA contents were subjected to LSC. (D) In the same experiment, the DNA contents of 100 karyomere-like cells were measured. Cell numbers in each DNA content range were plotted with histograms.

be surrounded by a nuclear envelope after chromosome segregation occurs. Therefore, those two types of abnormal structures are referred to as karyomere-like nuclei hereafter.

In addition to DNA damage, formation of karyomere-like nuclei may also occur as a consequence of dysfunctions of the mitotic apparatus [13,14]. Moreover, a previous study found that the anti-POLD4 antibody bound the surface of mitotic chromosomes, which suggests specific functions of POLD4 during mitosis [5]. To investigate this, we analyzed the centrosome structures in si *POLD4*-treated cells, as it has been reported that disturbed chromosomal migration occurred with abnormal replication or localization of centrosomes [15,16]. Our present results showed that si *POLD4*-treated cells were associated with normal centrosome structures, which had one or two centrosomes located at a single site (Fig. 2E). We also quantified the lagging-chromosome frequencies, and found that they were very similar between si*POLD4*- and si*CTRL*-treated mitotic cells (data not shown). Although the results of this limited experiment were contrary to our speculation that POLD4 has some mitotic functions, we intend to conduct more detailed examinations in the future.

Cell cycle dynamics of karyomere-like cells

In the following experiments, we studied the cell cycle dynamics of karyomere-like cells. After si*POLD4* treatment, we observed checkpoint activation (data not shown, detailed mechanisms discussed elsewhere), and accumulations of G1- and G2-populations (Fig. 3A). In si*CTRL*-treated cells, most of the karyomere-like populations were found among the minor aneuploid populations (Figs. 3B and 2F, left panels). In contrast, karyomere-like cells in si *POLD4*-treated cells were found to have normal ploidy as seen with 2N–4N cells (Fig. 3B, 2F, right panels).

In order to determine if karyomere-like cells remained alive and had an ability to progress through the cell cycle, we synchronized cells at the G1–S boundary, then released them and observed the cell cycle progression, as well as the associated nuclear shapes (Fig. 3C–E). Interestingly, karyomere-like cells progressed through the cell cycle and returned to G1 at 12 h after release. In support of these results, most karyomere-like cells were negative in TUNEL staining findings (data not shown). Therefore, these structures may not reflect the pro-apoptotic phenotype. Our results suggest that most karyomere-like cells are able to proliferate until they became arrested at the G1 or G2 phase, when DNA damage reaches an intolerant level.

In conclusion, our results showed that POLD4 supports cellular proliferation and suppresses karyomere-like nuclei formation in human cells, which might occur as a consequence of impairment of the DNA replication and repair activities of pol δ. A future study to identify the direct link between POLD4 and mitotic functions may reveal the underlying mechanisms to maintain genomic stability in human cells.

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