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Break-induced replication repair of damaged forks induces genomic duplications in human cells

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Abstract

In budding yeast, one-ended DNA double-strand breaks (DSBs) and damaged replication forks are repaired by break-induced replication (BIR), a homologous recombination pathway that requires the Pol32 subunit of DNA polymerase delta. DNA replication stress is prevalent in cancer, but BIR has not been characterized in mammals. In a cyclin E overexpression model of DNA replication stress, *POLD3*, the human ortholog of *POL32*, was required for cell cycle progression and processive DNA synthesis. Segmental genomic duplications induced by cyclin E overexpression were also dependent on *POLD3*, as were BIR-mediated recombination events captured with a specialized DSB repair assay. We propose that BIR repairs damaged replication forks in mammals, accounting for the high frequency of genomic duplications in human cancers.

Activated oncogenes induce collapse and/or breakage of DNA replication forks (damaged forks), leading to DNA replication stress and DNA DSBs (1, 2). To identify repair pathways for damaged forks, we performed an siRNA screen monitoring DNA synthesis in a U2OS cell system, in which tetracycline withdrawal induces cyclin E overexpression and, subsequently, DNA replication stress (3, 4). The siRNA library targeted 690 genes implicated in DNA metabolism and the cells were cultured with or without tetracycline and

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also with or without hydroxyurea (HU), to compare the responses to damaged versus stalled forks (5).

The screened genes were distributed into four clusters (fig. S1 and table S1). The first cluster, comprising genes important for DNA synthesis specifically in cells overexpressing cyclin E, included *POLD3*, which encodes a subunit of DNA polymerase delta (6), and homologous recombination (HR) genes (7). The second cluster mediated the response to HU and included the *BLM* helicase and the *ATR* and *CHK1* checkpoint genes (8, 9). The third cluster included the genes, whose depletion affected preferentially the cells exposed to HU and also overexpressing cyclin E. However, no gene in this cluster had a strong phenotype. Finally, the last cluster encompassed the genes, whose depletion did not affect the response to DNA replication stress.

From the identified hits, we pursued *POLD3*, whose budding yeast ortholog, *POL32*, is essential for BIR (10–12) (fig. S2). We also examined *POLD4*, which encodes the fourth subunit of DNA polymerase delta (6), since, in fission yeast, the orthologs of *POLD3* and *POLD4* are both non-essential (13). Cells expressing normal or high levels of cyclin E were transfected with siRNA and, three days later, exposed to two thymidine-analog pulses (EdU and BrdU, respectively; 1 h each, separated by 6 h) to monitor cell cycle progression (Fig. 1A and fig. S3). As reported (14), cyclin E overexpression enhanced the fraction of G1 cells entering S phase during the 8 h period (Fig. 1A and fig. S4). Depletion of *POLD3* or *POLD4* inhibited S phase entry in the cells overexpressing cyclin E, but had no effect in cells expressing normal cyclin E levels (Fig. 1A and fig. S5). In a similar assay, depletion of *POLD3* or *POLD4* had no effect on S phase entry of cells treated with HU or aphidicolin (fig. S6). Since short-term exposure to HU or aphidicolin induces fork stalling, but not fork damage (15), we conclude that the functions of *POLD3* and *POLD4* relate to damaged forks.

POLD3 or *POLD4* depletion also inhibited growth of U2OS cells overexpressing cyclin E ($P<0.001$), whereas growth of cells expressing normal cyclin E levels was unaffected (Fig. 1B and fig. S7). Growth of SAOS2 osteosarcoma, HeLa cervical carcinoma and MDA-MB157 breast carcinoma cells, all of which have DNA replication stress, was also inhibited following POLD4 depletion ($P<0.001$ for all), whereas growth of non-transformed cells, such as BJ fibroblasts and MCF10A mammary epithelial cells, was unaffected (Fig. 1B).

Next, we analysed replication forks by DNA combing. In U2OS cells expressing normal cyclin E levels, most of the forks were ongoing (about 60%), irrespective of *POLD3* or *POLD4* depletion. In cells overexpressing cyclin E, the fraction of ongoing forks was still higher (about 45%), than the fraction of terminated forks (about 28%). However, when *POLD3* or *POLD4* were depleted, the ongoing forks became a minority (about 17%) and the terminated forks the majority (about 47%), suggesting that *POLD3* and *POLD4* are important for fork processivity when cyclin E is overexpressed (Fig. 2A). As reported (16), cyclin E overexpression reduced replication fork speeds (Fig. 2B and fig. S8). Depletion of *POLD3* or *POLD4* did not affect fork speeds in cells with normal cyclin E levels, but in the cells overexpressing cyclin E, the forks traveling slower than 0.5 kb/min were preferentially targeted ($P<0.005$; Fig. 2B). Thus, slow forks may be different from fast forks.

In budding yeast, BIR repairs damaged replication forks, but also one-ended DNA DSBs (11, 12). To explore a role of *POLD3* and *POLD4* in DSB repair, various human cell lines transfected with siRNA were exposed to ionizing radiation (IR) and 53BP1 and RPA foci, surrogate markers for unrepaired DNA DSBs and DNA replication stress, respectively (17), were scored. Both types of foci persisted longer in the cells, in which *POLD3* or *POLD4* were depleted (Fig. 3A and fig. S9; U2OS cells: $P<0.01$ for 53BP1 foci at 16 h and for RPA foci at 24 h).

The role of *POLD3* and *POLD4* in DNA DSB repair was further examined using GFP-based reporters, in which DSBs were induced by the nuclease I-SceI. The reporters monitoring synthesis-dependent strand annealing (SDSA) and single-strand annealing (SSA) (18) were supplemented with a newly-developed BIR reporter, in which the sequence homology was only on one side of the DSB, to prevent repair by SDSA, and the homologous *GFP* sequences were in opposite orientations, to prevent repair by SSA (Fig. 3B). Repair of the I-SceI-induced DSB by BIR would start with invasion of the broken end into an uncut homologous template (Fig. 3Ci). Conservative replication initiated from the invading strand (19) would restore the *GFP* coding sequence (Fig. 3Cii). Then, the low processivity of DNA polymerase delta, which is the polymerase at the invading strand (20), would lead to replication fork disengagement and the newly-created DNA end would be joined to the only other available free end, the one generated by I-SceI (Fig. 3Ciii).

The BIR reporter described above was stably-integrated in U2OS cells and DSBs were induced by expressing I-SceI. Sequencing of PCR products prepared using primers specific for *GFP* (primers P5GFP and P3GFP, respectively; Fig. 3C) showed accurate recombination. Next, the predicted end joining-generated junctions were amplified using forward primers downstream of *GFP* (primers P5EJ1, P5EJ2 or p5EJ3) and a reverse primer downstream of the I-SceI cleavage site (primer P3EJ; Fig. 3C). The sequences of eight breakpoint junctions were consistent with stochastic dissociation of the BIR-initiated fork from the template and with variable resection at the I-SceI-induced break (Fig. 3Ciii). Microhomologies (2–4 bps) or small insertions were present in all breakpoint junctions (fig. S10), suggesting repair of the free ends by microhomology-mediated end joining (MMEJ, also known as backup-EJ or Alt-EJ), a Ku-independent break repair mechanism (21). Thus, human cells can repair one-ended DNA DSBs by BIR.

Depletion of *POLD3*, suppressed DSB repair by BIR ($P<0.002$), but did not affect repair by SDSA or SSA (Fig. 3D), consistent with *POLD3* mediating processive DNA synthesis at the invading strand, as shown for yeast and *Drosophila POL32* (20, 22). In contrast, depletion of *POLD4* had no effect in any of the repair assays (Fig. 3D). Both *POLD3* and *POLD4* contain PCNA-binding motifs and both enhance DNA polymerase delta processivity *in vitro* (6, 23), but, perhaps, *POLD4* is less active than *POLD3*. In our assay, DNA synthesis of as little as 1 kb conferred GFP expression (Fig. 3C), but in *Drosophila*, the effects of *POL32* on extension of the invading strand are barely evident, when synthesis is limited to 1 kb (22).

In budding yeast, *POL32* is required for induction of tandem duplications under conditions of DNA replication stress and these duplications were attributed to BIR or to

microhomology-induced replication, a BIR-related mechanism (24). In fission yeast, duplications associated with fold-back inversions have also been attributed to BIR (25). By array-based comparative genomic hybridization (CGH), overexpression of cyclin E in U2OS cells for three weeks induced copy number alterations (CNAs) (Fig. 4A; $P<0.05$, number of CNAs for cyclin E overexpression versus normal expression). To examine the effect of depleting *POLD3* or *POLD4* on the spectrum of CNAs we repeated the experiment, except that during the period of cyclin E overexpression, the cells were transfected every three days with siRNA. In single cell clones isolated from cells transfected with control siRNA, amplifications (presumably, duplications) less than 200 kb long accounted for a third of all CNAs (Fig. 4B and table S2). However, in clones isolated from *POLD3*- or *POLD4*-depleted cells, the frequency of such amplifications decreased by half (Fig. 4B; $P<0.02$, control versus *POLD3* siRNA; $P<0.08$, control versus *POLD4* siRNA; $P<0.01$, control versus *POLD3* siRNA and *POLD4* siRNA grouped together). In yeast, BIR can lead to DNA synthesis of about 100 kb (26). Thus, duplications of up to 200 kb in human cells may represent BIR events, whereas the larger amplifications and deletions may arise from other repair mechanisms, such as non-allelic homologous recombination.

PCR primers designed using the CGH array data failed to amplify CNA breakpoint junctions. Thus, genomic DNA from two clones was subjected to paired-end high-throughput sequencing. In the first clone, derived from cells transfected with control siRNA, five junctions were identified (Fig. 4C and figs S11 and S12), revealing two head-to-tail tandem duplications, one duplication associated with a fold-back inversion, and two simple deletions. Microhomologies were present in all breakpoint junctions. In the second clone, derived from cells transfected with *POLD3* siRNA, two junctions were identified, revealing two head-to-tail tandem duplications, of which one had a microhomology junction and the other a 5 bp insertion (Fig. 4C and fig. S12).

The most frequent type of CNA in breast and ovarian cancers, representing one third of all somatic rearrangements, is tandem head-to-tail duplications with microhomology junctions (27, 28). Fold-back inversions are very common in pancreatic cancer (29). Both types of CNAs were observed in U2OS cells overexpressing cyclin E and their frequency decreased after depleting *POLD3* or *POLD4*. Thus, BIR repair of damaged replication forks might explain the presence of segmental genomic duplications in human cancers (fig. S13). Notably, the *POLD3* gene is frequently amplified in human cancers (30) and its protein product is overexpressed in cancer cell lines (fig. S14).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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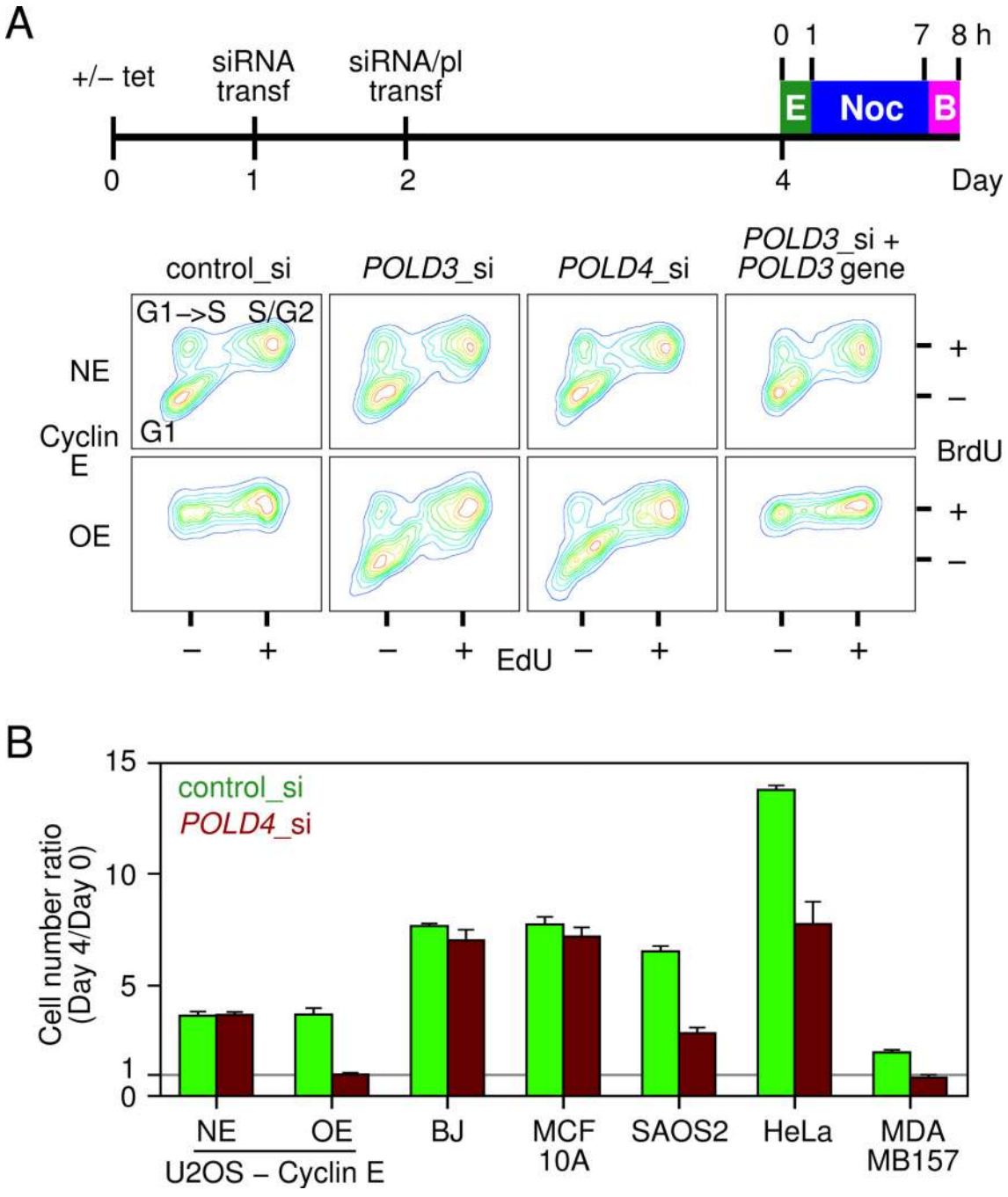


Fig. 1. *POLD3* and *POLD4* are required for cell cycle progression in the presence of oncogene-induced DNA replication stress

(**A**) Top, experimental outline. Bottom, flow cytometry analysis. U2OS cells expressed normal levels of cyclin E (NE) or overexpressed cyclin E (OE). E, EdU; B, BrdU; Noc, nocodazole; si, siRNA; EdU–/BrdU–, cells that remained in G1 or were blocked at the G1/S interface (G1); Edu–/BrdU+, cells that were in G1 at 0–1 h, but entered S phase by 7–8 h (G1->S); EdU+/BrdU+, cells that were in S phase at 0–1 h (S/G2).

(**B**) Effect of *POLD4* depletion on cell growth. The cells were seeded on Day 0; transfected with siRNA on Day 1 and counted on Day 4. Means and SDs from three independent experiments are shown.

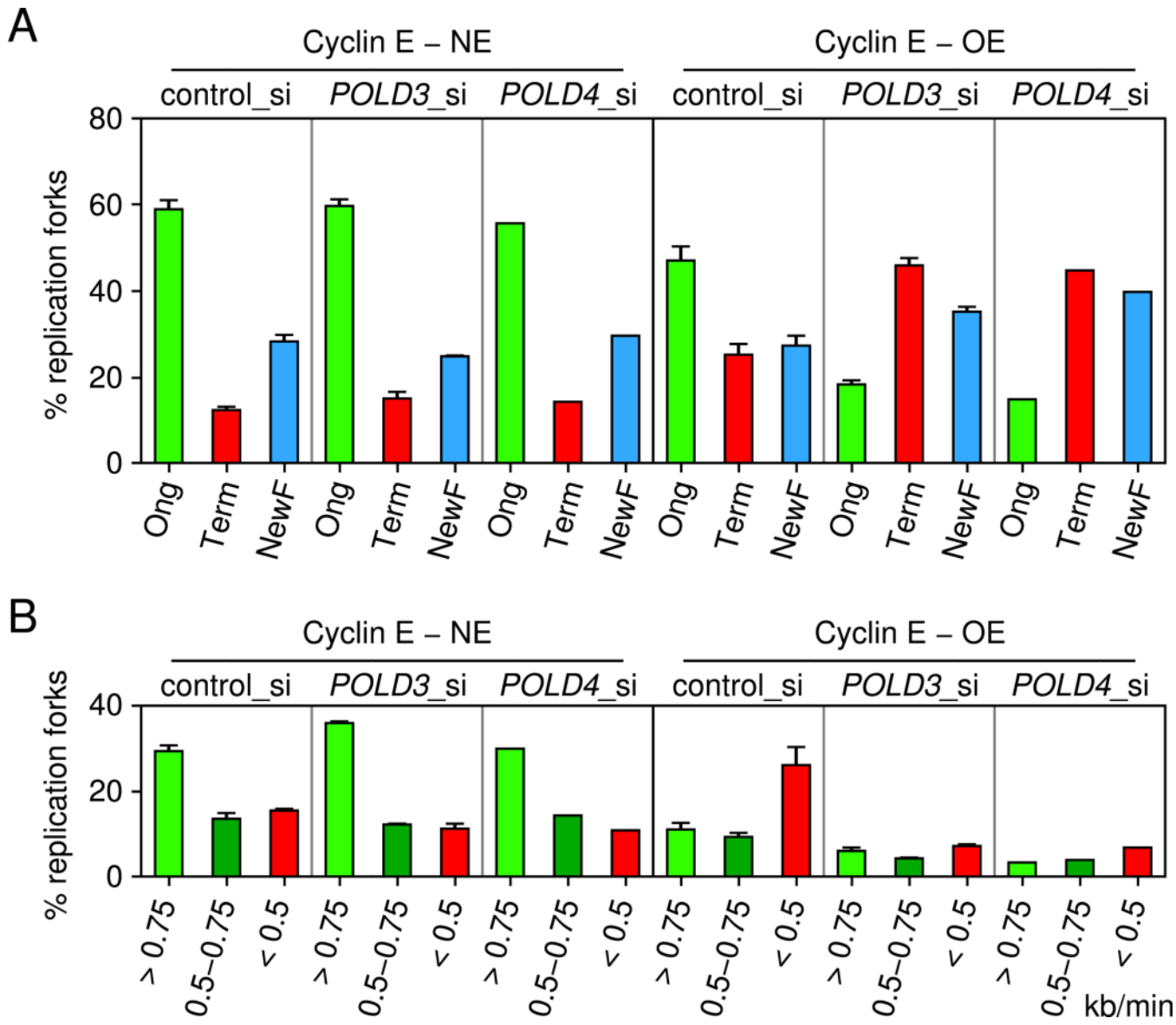


Fig. 2. *POLD3* and *POLD4* are required for fork processivity under conditions of oncogene-induced DNA replication stress

(**A**) Distribution of replication forks. U2OS cells expressed normal levels of cyclin E (NE) or overexpressed cyclin E (OE). Replication forks were scored as ongoing (Ong), terminated (Term) or newly-fired (NewF). The data represent two independent experiments for *POLD3* depletion and one experiment for *POLD4* depletion. si, siRNA.

(**B**) Distribution of replication speeds of ongoing DNA replication forks as a function of cyclin E expression levels and *POLD3/POLD4* depletion. The percentages are relative to the total number of forks counted (ongoing, terminated and newly-fired), but only data for the ongoing forks are presented.

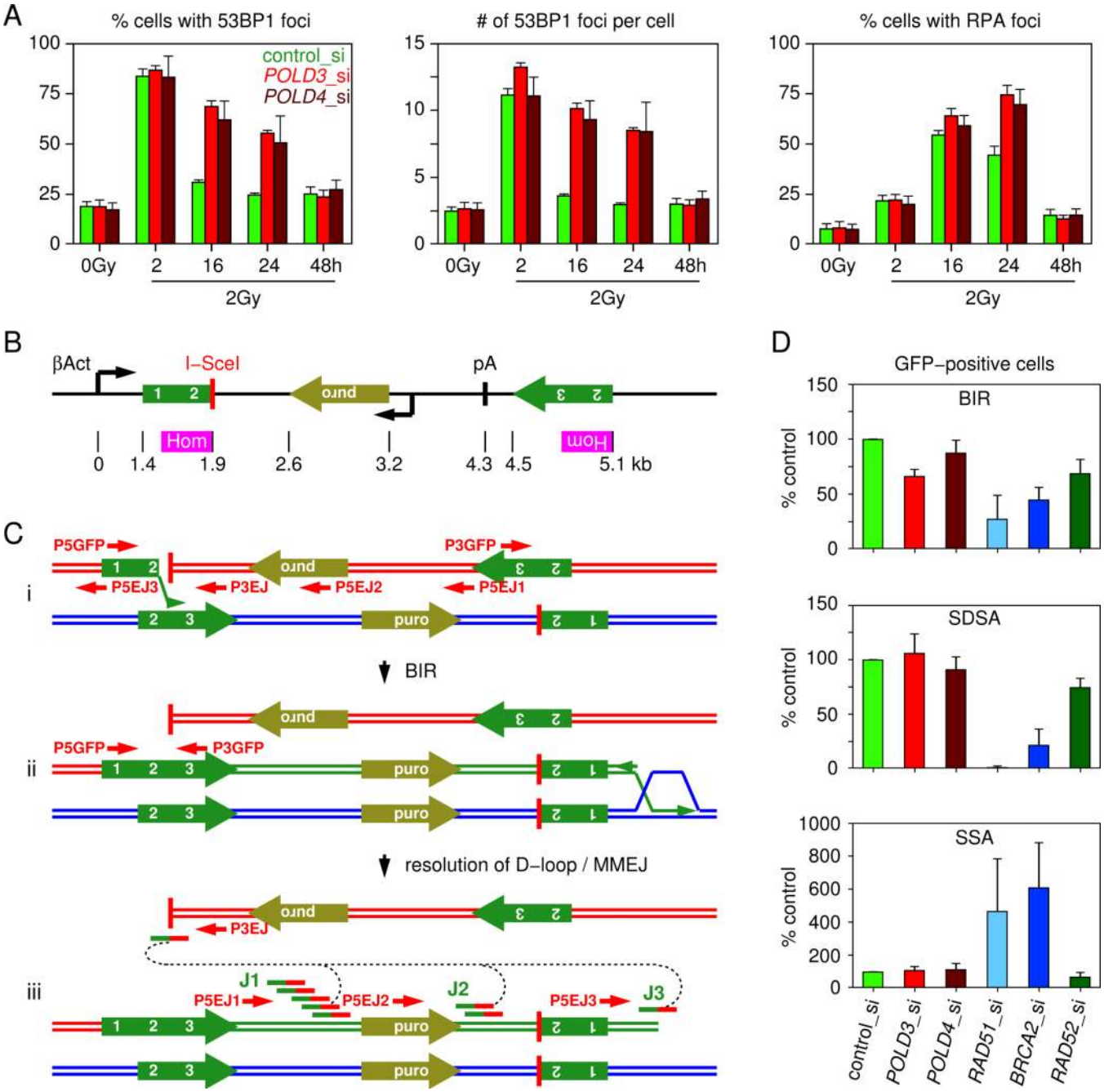
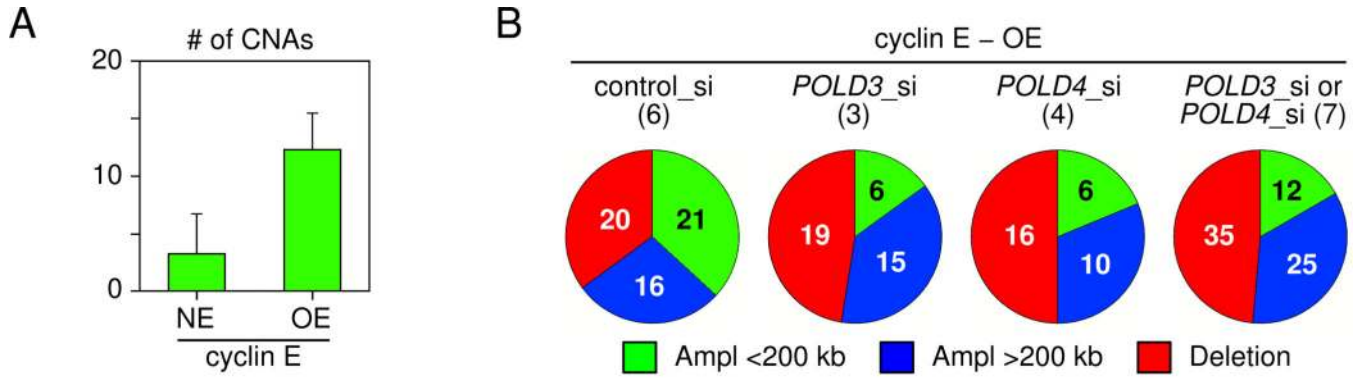


Fig. 3. Role of *POLD3* and *POLD4* in DNA DSB repair

(**A**) Percentages of cells with 53BP1 or RPA foci and average number of 53BP1 foci per cell in non-irradiated (0 Gy) and irradiated (2 Gy; 2, 16, 24 and 48 h after irradiation) U2OS cells. si, siRNA.

(**B**) GFP-based reporter plasmid to monitor BIR. A beta-actin (βAct) promoter drives expression of N-terminal *GFP* sequences. The C-terminal *GFP* sequences are in the opposite orientation. The area of homology (Hom, 400 bps) is limited to one side of the I-SceI-induced break. Position 0 kb refers to the beta-actin promoter transcription start site. pA, poly A site.

(**C**) Expected and observed products following repair by BIR. Three steps are shown: i) homology search; ii) strand extension; iii) dissolution of the D-loop and joining of the free DNA ends by MMEJ. P5GFP and P3GFP, primers to amplify *GFP*; P5EJ1, P5EJ2, P5EJ3 and P3EJ, primers to amplify MMEJ-generated junctions of types J1, J2 and J3, respectively. The short green/red lines indicate eight observed junctions.
(**D**) Effect of *POLD3* or *POLD4* depletion on repair of DNA DSBs by BIR, SDSA and SSA. Means and SDs from experiments performed in quadriplicate are relative to control siRNA-transfected cells. si, siRNA.



C

		Chr.	CNA	Junction (position)	Junction (sequence)
cyclin E – OE	control_si	11	tand. dupl. (109 kb)	8709669/8600962	CGGGCTGTA AAG AATCTCAGA
		14	tand. dupl. (160 kb)	51446913/51286845	TGTGAAATG ACA TGAAATCAA
		X	ampl./ inv. (3.7 kb)	100377888/100374143	CAGCAAGAT CT GGGCAACAA
		11	deletion (178 kb)	83241462/83419178	CTTTTTTTT TT ATGATAATT
		16	deletion (623 kb)	66542790/67165475	TATTGGAAC AAG CCAGACCTC
	*POLD3*_si	1	tand. dupl. (43 kb)	79871112/79827707	TATATTTGG ACCT TCCATATGT
		10	tand. dupl. (81 kb)	67706779/67626247	ATTAAAGTC TTGTT CCATTTTAA

Fig. 4. Cyclin E overexpression-induced genomic rearrangements

(**A**) Number of copy number alterations (CNAs) induced over a three week period in U2OS cells expressing normal or high levels of cyclin E (NE and OE, respectively). Means and SDs are from three clones per group.

(**B**) Number of different types of CNAs (amplifications smaller than 200 kb, amplifications greater than 200 kb and deletions) induced in U2OS cells overexpressing cyclin E over a three week period, during which, the cells were transfected every three days with siRNA. The number of cell clones analysed is in parentheses. The data from the *POLD3* and *POLD4*-depleted clones are also shown grouped together. si, siRNA.

(**C**) Sequences of breakpoint junctions in two cyclin E-overexpressing clones, of which one had been transfected with control siRNA and one with siRNA targeting *POLD3*. The type of CNA associated with these junctions, the size of the CNA and the affected chromosome are indicated. The two joined sequences are colored blue and red, respectively; the microhomologies or small insertions at the junction are colored purple and brown, respectively.