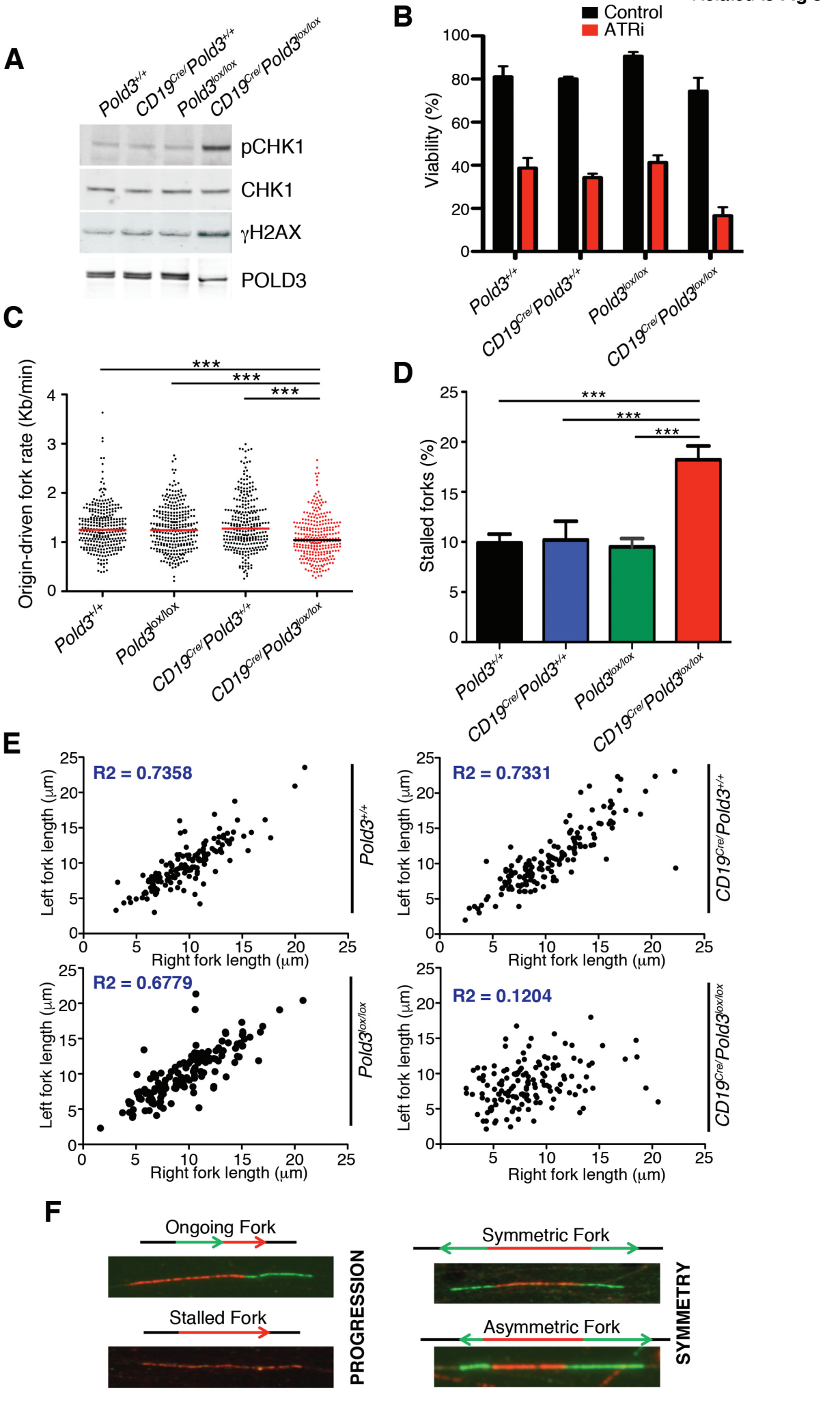
A
Pold3+/+
CD19Cre/Pold3+/+
Pold3lox/lox
CD19Cre/Pold3lox/lox
pCHK1
CHK1
γH2AX
POLD3
B
Control
ATRi
Viability (%)
100
80
60
40
20
0
Pold3+/+
CD19Cre/Pold3+/+
Pold3lox/lox
CD19Cre/Pold3lox/lox
C
Origin-driven fork rate (Kb/min)
***
***
***
4
3
2
1
0
Pold3+/+
Pold3lox/lox
CD19Cre/Pold3+/+
CD19Cre/Pold3lox/lox
D
Stalled forks (%)
***
***
***
25
20
15
10
5
0
Pold3+/+
CD19Cre/Pold3+/+
Pold3lox/lox
CD19Cre/Pold3lox/lox
E
R2 = 0.7358
Pold3+/+
R2 = 0.7331
CD19Cre/Pold3+/+
R2 = 0.6779
Pold3lox/lox
R2 = 0.1204
CD19Cre/Pold3lox/lox
Left fork length (μm)
Right fork length (μm)
0
5
10
15
20
25
F
Ongoing Fork
Stalled Fork
PROGRESSION
Symmetric Fork
Asymmetric Fork
SYMMETRY

Figure S1 (*related to Fig. 3*). (A) WB illustrating the levels of phosphorylated CHK1 (S345), total CHK1, γH2AX and POLD3 in B cells from the indicated genotypes, 48 hrs after stimulation with LPS (25 μg/ml). (B) Percentage of viable cells (measured by FACS) in B cell cultures of the indicated genotypes, 48 hrs after stimulation with LPS (25 μg/ml) in the presence or absence of a previously described ATR inhibitor (Toledo et al. 2011) for the last 24 hrs (5 μM). Data are represented as mean ± SD (n=3). (C) Fork rates were measured in stretched DNA fibers prepared from B cells of the indicated genotypes. The data used for the analysis are the same ones shown in Figure 3E, but restricting the analysis to forks are derived from active replication origins ("green-red-green" tracks or two proximal "red-green" forks moving in opposite directions in the same fiber). 296 (*Pold3*$^{+/+}$), 292 (*Pold3*$^{lox/lox}$), 288 (CD19^{Cre}/*Pold3*$^{+/+}$) and 288 (CD19^{Cre}/*Pold3*$^{lox/lox}$) tracks were used for the analysis in each case. ***P<0.001. (D) Percentage of stalled replication forks found by DNA fiber analyses (see Methods) from fibers prepared from B cell cultures of the indicated genotypes. Data are represented as mean ± SD (n=3). ***P<0.001. (E) Fork asymmetry (represented as left *vs* right fork lengths) in DNA fibers prepared from B cells of the indicated genotypes. The determination coefficient is indicated in blue (R2). (F) Examples of the types of figures that are encountered during the analyses of DNA fibers.

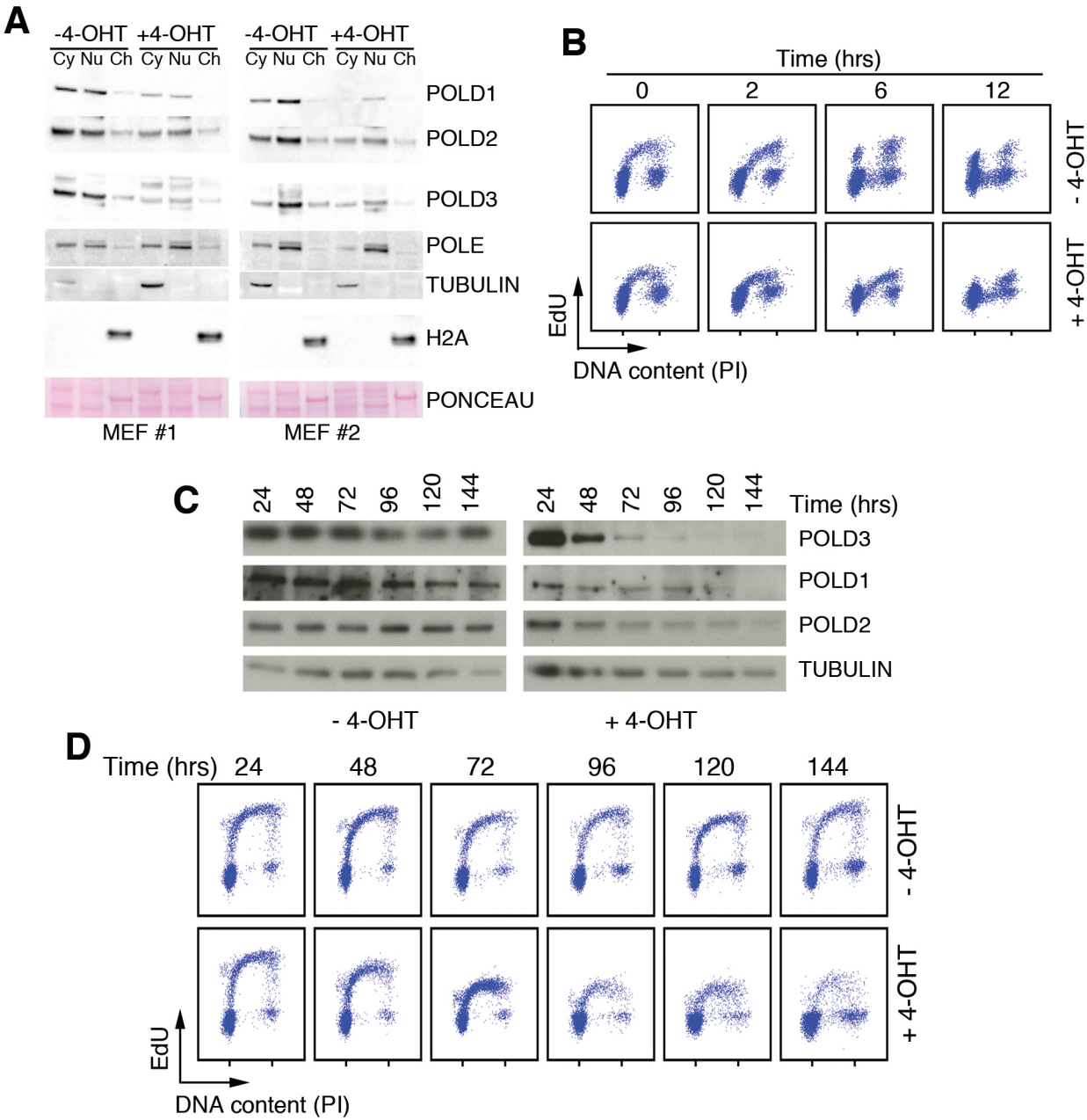
A
-4-OHT +4-OHT
Cy Nu Ch Cy Nu Ch
-4-OHT +4-OHT
Cy Nu Ch Cy Nu Ch
POLD1
POLD2
POLD3
POLE
TUBULIN
H2A
PONCEAU
MEF #1
MEF #2
B
Time (hrs)
0
2
6
12
- 4-OHT
+ 4-OHT
EdU
DNA content (PI)
C
24
48
72
96
120
144
Time (hrs)
POLD3
POLD1
POLD2
TUBULIN
- 4-OHT
+ 4-OHT
D
Time (hrs) 24
48
72
96
120
144
- 4-OHT
+ 4-OHT
EdU
DNA content (PI)

Figure S2 (*related to Fig. 4*). (A) WB illustrating the levels of POLD1, POLD2, POLD3 and POLE in cytoplasmic (Cy), nuclear (Nu) and chromatin (Ch) fractions from two independent Ub^{Cre}/*Pold3*$^{lox/lox}$ MEF exposed (or not) to 4-OHT (1 μM) for 72 hrs. TUBULIN and H2A are shown as controls for the fractionation, and the Ponceau image of the membrane to illustrate the overall protein levels in each fraction. (B) Ub^{Cre}/*Pold3*$^{lox/lox}$ MEFs were treated with 4-OHT or not treated and two days later pulsed-labeled for 1h with 10 μM EdU. After washing off the EdU, cells were allowed to proceed through S phase for the indicated time periods (0, 2, 6 or 12h) and analyzed by FACS. Note that while untreated MEF were able to progress through S-phase and enter the next G1 by 6 hrs, *Pold3*-deleted cells failed to do so even at 12 hrs after the EdU pulse. (C) WB illustrating the levels of POLD1, POLD2 and POLD3 in Ub^{Cre}/*Pold3*$^{lox/lox}$ MEF exposed (or not) to 4-OHT for the indicated times. TUBULIN levels are shown as loading control. (D) DNA replication was analysed by monitoring the amount of EdU incorporation (10 μM, 1hr) by FACS in Ub^{Cre}/*Pold3*$^{lox/lox}$ MEF treated as in (C) for up to 7 days. Note the progressive decrease in EdU incorporation rates which correlates with the decrease in POLD1-3 levels shown in (C).

Fig S3
Related to **Fig 4**

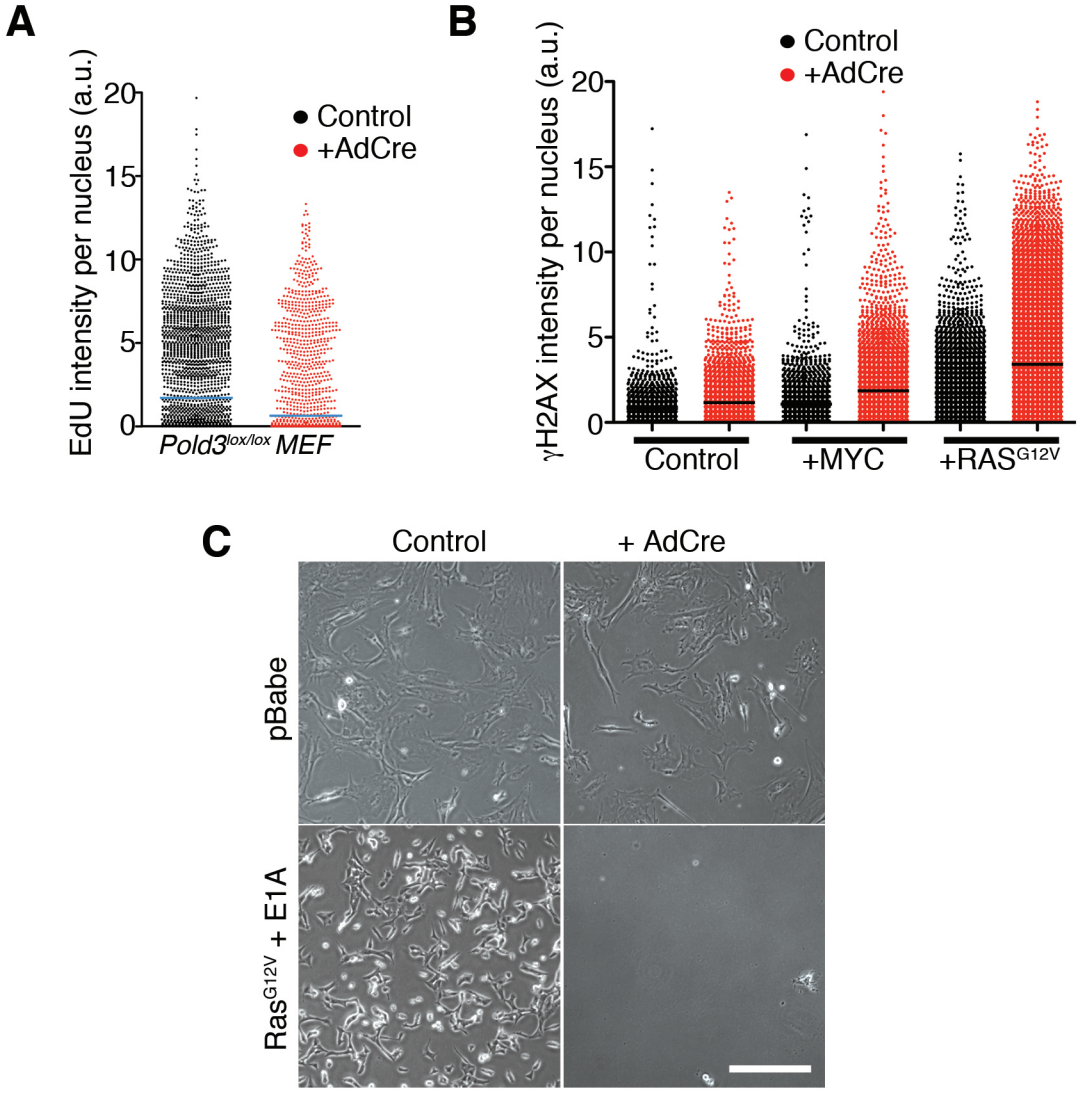

Figure S3 (*related to* ***Fig. 4***). (A) DNA replication rates from *Pold3*$^{lox/lox}$ MEF infected with AdCre (or mock infected), as measured by HTM quantifying the levels of EdU incorporation per individual nucleus. EdU was added at 10 μM for 30 min. (B) The impact of POLD3 deficiency in oncogene-induced DNA damage was evaluated by infecting *Pold3*$^{lox/lox}$ MEF with retroviruses expressing MYC or RAS^{G12V} and E1A oncogenes (or with a control retroviral vector, pBabe). 48 hrs after infection with oncogene-expressing viruses, cells were exposed to AdCre for another 24 hrs, after which the levels of γH2AX per individual nucleus were analyzed by HTM. (C) Representative pictures from the experiment quantified in (B). Images were taken 48 hrs after infection with AdCre.

SUPPLEMENTAL TABLES

Table S1, related to Fig 4. Proteomic analyses of POLD3 immunoprecipitation from WT and POLD3-deficient B cells.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animal research

The linearized *Pold3*$^{GT:lox}$ vector (EUCOMM) was used for the electroporation of mouse ES cells. Recombinant ES cells were screened by Southern Blot through standard procedures, and subsequently used for the generation of chimaeras by aggregation. *Pold3* mutant strains were genotyped by PCR (primers available upon request). CD19^{Cre}, Ub^{Cre}, EIIA-Cre and actin-FLP strains have been described before (Lakso et al. 1996; Rickert et al. 1997; Rodriguez et al. 2000; Ruzankina et al. 2007). Mice were kept under standard conditions at specific-pathogen free facility of the Spanish National Cancer Centre in a mixed C57BL/6-129/Sv background. All mouse work was performed in accordance with the Guidelines for Humane Endpoints for Animals Used in Biomedical Research, and under the supervision of the Ethics Committee for Animal Research of the "Instituto de Salud Carlos III".

Inmunohistochemistry

Tissues were fixed in formalin and embedded in paraffin for subsequent processing. Consecutive 2.5-μm sections were treated with citrate for antigenic recovery and processed for immunohistochemistry with an antibody against γH2AX (Millipore, 05-636). IHCs were scanned and digitalized with a MIRAX system (Zeiss) for further analysis.

Cell culture

MEF from E13.5 d.p.c. embryos were generated by standard methods and grown in Dulbecco's Minimum Essential Media (DMEM, Invitrogen) supplemented with 15% FBS (Lonza). For all experiments, MEF were used at a low passage (<3) and grown in 5% oxygen to minimize exposure to reactive oxygen species. For B cell cultures, resting splenic B cells were isolated with anti-CD43 microbeads (anti-Ly48; Miltenyi Biotech) and cultured in the presence of LPS (25 μg/ml; Sigma). For the analysis of chromosomal rearrangements, colcemide (Sigma) was added at 0.1 μg/ml for the last 5 hrs and metaphases prepared using a standard hypotonic/Carnoy procedure followed by Giemsa staining.

High Throughput Microscopy

MEF were grown on μCLEAR bottom 96-well plates (Greiner Bio-One). Immunofluorescence was performed using standard procedures with an antibody against γH2AX (Millipore). Analysis of DNA Replication by EdU incorporation was done using click-it (Invitrogen) following manufacturers instructions. In all cases, images were automatically acquired from each well using an Opera High-Content Screening System (Perkin Elmer). A 20x magnification lens was used and images were taken at non-saturating conditions. Images were segmented using the DAPI signal to generate masks matching cell nuclei from which the mean signals for the rest of the stainings (γH2AX or EdU) were calculated. Data were represented with the use of the Prism software (GraphPad Software).

Immunoblotting

For protein extracts, cells were washed once with PBS, and lysed in RIPA buffer (Tris-HCl 50 mM, pH 7.4, NP-40 1%, Na-deoxycholate 0.25%, NaCl: 150 mM, EDTA 1 mM) containing protease and phosphatase inhibitors (Sigma). Samples were resolved by SDS-PAGE and analyzed by standard Western blotting techniques. Antibodies against CHK1 (Novocastra, NCL-Chk1), Chk1-S345P (Cell Signaling, 2348S), H2A (Cell Signaling, #3636), γH2AX (Millipore, 05-636), CDK2 (Santa Cruz, M2, sc-163), POLD1 (Santa Cruz, sc373731), POLD2 (Sigma, HPA026745), POLD3 (Sigma, HPA039627), POLE (GeneTex, GTX113806), TUBULIN (Sigma, T9026) and β-ACTIN (Sigma, A5441) were used. Protein blot analyses were performed on the LICOR platform (Biosciences).

DNA fiber analyses

Exponentially growing cells were pulse-labeled with 50 μM CldU (20 min) followed by 250 μM IdU (20 min). Labeled cells were collected and DNA fibers were spread in buffer containing 0.5% SDS, 200 mM Tris pH 7.4 and 50 mM EDTA. For immunodetection of labeled tracks, fibers were incubated with primary antibodies (for CldU, rat anti-BrdU (Abcam, ab6326); for IdU, mouse anti-BrdU (BD Biosciences, 347580)) and developed with the corresponding secondary antibodies conjugated to Alexa dyes. Mouse anti-ssDNA antibody (Millipore, MAB3034) was used to assess fiber integrity. Slides were examined with a Leica DM6000 B microscope, as described previously (Mouron et al. 2013). The conversion factor used was 1 μm = 2.59 kb (Jackson and Pombo 1998). When indicated, analysis of fork rate was restricted to those forks that are positively derived from active replication origins. In this case, the length of green (IdU) tracks was measured only when included in 'green-red-green' tracks or in two proximal 'red-green' forks that are moving in opposite directions in the same fiber.

POLD3 immunoprecipitation followed by Mass Spectrometry

Protein G-Dynabeads were washed twice in 50 mM Tris, pH 7.9, and 200 mM NaCl, incubated with specific antibodies in the presence of 1 mg/ml BSA for 1h at 4ºC and washed 5 times in 50 mM Tris, pH 7.9, and 200 mM NaCl before the incubation with the extract. 500 μg nuclear extract at 1 mg/ml in 50 mM Tris, pH 7.9, and 200 mM NaCl was centrifuged for 10 min at 20000 g at 4°C. The supernatant was incubated with Protein G-Dynabeads coated with the specific antibodies at 4°C overnight. The beads were washed 5 times with 50 mM Tris, pH 7.9, 200 mM NaCl and 0.05% Igepal CA630 (Sigma). Proteins were eluted in 8 M urea in 0.1 M triethylammonium bicarbonate TEAB (UA) and digested using the FASP method (Wisniewski et al. 2009) with some modifications. Briefly, samples were loaded onto centrifugal devices Microcon-30 (Merck Millipore). Filters were washed with UA and proteins were alkylated using 50 mM iodoacetamide for 20 min in the dark. Proteins were digested during 5 h with endoproteinase Lys-C (Wako). Finally, trypsin (Promega, Madison, WI) was added and samples were subjected to an overnight digestion. Reaction was quenched by acidification (final TFA concentration of 0.5%).

Peptides were desalted with home-made Stage-tips using Poros R3 resin, dried down in a speed-vac and re-suspended in 22 μL of 0.1% of formic acid.

NanoLC-MS/MS was performed using an LTQ-Orbitrap Velos (Thermo Scientific) coupled online to a nanoLC Ultra system (Eksigent), equipped with a nanoelectrospray ion source (Proxeon Biosystems). Samples (10 μL) were loaded onto a reversed-phase C18, 5 μm, 0.1 x 20 mm trapping column (NanoSeparations) and washed for 15 min at 2.5 μl/min with 0.1% FA. The peptides were eluted at a flow rate of 300 nl/min onto a home-made analytical column packed with ReproSil-Pur C18-AQ beads, 3 µm, 75 µm x 50 cm, heated to 45 °C. Solvent A was 4% ACN in 0.1% FA and Solvent B acetonitrile in 0.1% FA. The following gradient was used: 0–2 min 2% B, 2–103 min 6–35% B, 103–103.5 min 35-98% B, 103.5–113.5 min 98% B, 114–120 min 2% B The LTQ Orbitrap Velos was operated in a data dependent mode. The spray voltage was set to 1.8 kV and the temperature of the heated capillary was set to 325^0C. The MS survey scan was performed in the FT analyzer scanning a window between 350 and 1500 *m*/*z*. The resolution was set to 60 000 FWHM at *m/z* 400. The *m*/*z* values triggering MS/MS with a repeat count of 1 were put on an exclusion list for 60 s. The minimum MS signal for triggering MS/MS was set to 800 counts. In all cases, one microscan was recorded. The 15 most abundant isotope patterns with charge ≥2 from the survey scan were sequentially isolated with an isolation window of 1.5 *m*/*z* and fragmented in the linear ion trap (LTQ) by collision induced dissociation (CID) using a normalized collision energy of 35%. The Q value to 0.25 and an activation time to 10 ms. The maximum ion injection times for the survey scan and the MS/MS scans were 500 ms and 100 ms respectively and the ion target values were set to 1e6 and 5000, respectively for each scan mode.

Raw data was processed using Proteome Discoverer 1.4 (Thermo) with Sequest HT as the search engine against a UniprotKB/TrEMBL database of *Mus musculus* (43,937 sequences) supplemented with frequently observed contaminants (397 sequences). Carbamidomethylation of cysteine was set as fixed modification and oxidation of methionine as variable modification. Precursor mass tolerance was 20 ppm and fragment mass tolerance was 0.5 Da. Peptide Spectrum Matches (PSMs) were filtered using Percolator (v2.04) with a FDR of 1%.

ANTIBODIES USED IN THIS WORK:

Antibody	Use	Dilution	Reference
CHK1	Western blot	1/500	Novocastra, NCL-Chk1
CHK1-S345P	Western blot	1/500	Cell Signaling, #2348S
H2A	Western blot	1/1000	Cell Signaling, #3636
γH2AX	Western blot	1/1000	Millipore 05-636
CDK2	Western blot	1/2000	Santa Cruz, M2, sc-163
POLD1	Western blot	1/200	Santa Cruz, sc373731
POLD2	Western blot	1/1000	Sigma, HPA026745
POLD3	Western blot	1/1000	Sigma, HPA039627
POLE	Western blot	1/1000	GeneTex, GTX113806
α-Tubulin	Western blot	1/50000	Sigma, T9026
β-Actin	Western blot	1/50000	Sigma, A5441
CldU	Fiber analysis	1/100	Abcam ab6326
IdU	Fiber analysis	1/150	BD Biosciences 347580
ssDNA	Fiber analysis	1/200	Millipore MAB3034
POLD3	Immunoprecipitation	3.5 μg/500 μg extract	Sigma, HPA039627
γH2AX	IHC	1/1000	Millipore 05-636

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