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Rev3, the catalytic subunit of Polζ, is required for maintaining fragile site stability in human cells

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ABSTRACT

It has been long speculated that mammalian Rev3 plays an important, yet unknown role(s) during mammalian development, as deletion of *Rev3* causes embryonic lethality in mice, whereas no other translesion DNA synthesis polymerases studied to date are required for mouse embryo development. Here, we report that both subunits of Polζ (Rev3 and Rev7) show an unexpected increase in expression during G_2/M phase, but they localize independently in mitotic cells. Experimental depletion of Rev3 results in a significant increase in anaphase bridges, chromosomal breaks/gaps and common fragile site (CFS) expression, whereas Rev7 depletion primarily causes lagging chromosome defect with no sign of CFS expression. The genomic instability induced by Rev3 depletion seems to be related to replication stress, as it is further enhanced on aphidicolin treatment and results in increased metaphase-specific Fanconi anemia complementation group D type 2 (FANCD2) foci formation, as well as FANCD2-positive anaphase bridges. Indeed, a long-term depletion of Rev3 in cultured human cells results in massive genomic instability and severe cell cycle arrest. The aforementioned observations collectively support a notion that Rev3 is required for the efficient replication of CFSs during G_2/M phase, and that the resulting fragile site instability in *Rev3* knockout mice may trigger cell death during embryonic development.

INTRODUCTION

Recent studies support a notion that replication is incomplete within S-phase, and that many genomic loci known as late-replicating regions undergo replication well into the G_2/M phase (1–3). These late-replicating regions mostly have complex inherent nucleotide arrangement that often cause replication machinery to fail if a cell undergoes moderate replication stress, and they are expressed as gaps, constrictions and breaks (collectively referred to as breaks), which are also known as fragile sites (FSs) (4). One category, known as common fragile sites (CFSs), is of particular interest, as CFSs represent 'hot spots' of genomic instability, including chromosome breaks, translocations, deletions, sister chromatid exchanges, viral integration and gene amplification (5,6). Hence, CFS expression plays a critical role in genome instability, a hallmark of cancer. Indeed, the association between cancer and CFS instability was reaffirmed by recent studies (7–9), suggesting that CFS instability drives oncogenesis from the earliest stages. Even though fragile in nature, CFSs are highly stable and are expressed only when a cell undergoes replication stress, indicating that cells have developed an efficient mechanism to protect these otherwise unstable regions of the genome. To date, more than a dozen proteins have been implicated in the maintenance of CFSs (5,10,11), although it remains unclear how they function. Some recent studies have increased our understanding of the fragile nature of these genomic regions. For example, one mechanism is thought to be that the core region of CFSs lacks replication initiation events; therefore, it needs more time to complete replication (3). A recent study suggests that BLM is required to maintain a balanced pyrimidine pool and fork speed (12). Similar fork speed slowdown has also been reported with regard to some other proteins required for CFS maintenance, such as Claspin, checkpoint kinase 1 (CHK1) and Rad51, indicating that CFS expression is at least in part because of delayed completion of replication (5). Surprisingly, although CFS expression is primarily a defect in DNA replication, to date only a Y-family polymerase has been implicated in this event (13).

Polζ, which consists of the catalytic subunit Rev3 and the accessory subunit Rev7 (14), is the only known B-family translesion synthesis (TLS) polymerase. It is capable of bypassing certain DNA adducts efficiently

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(15–17), and more importantly, it is required for the extension step after nucleotide insertion by Y-family polymerases across from a replication-blocking lesion (18). Besides its role in TLS, *Rev3* is also essential for mouse embryonic development (19–21). This essential function is probably independent of TLS, as deletion of other TLS polymerase genes does not cause embryonic lethality (22–26). Rev3 has been implicated in homologous recombination repair (27–29); however, this activity is not unique to Rev3; hence, it is unlikely to provide the underlying mechanism of $Rev3^{-/-}$ embryonic lethality. Because of its extremely large size (>350 kDa), little progress has been made to understand the essential function of Rev3.

During the course of studying mammalian TLS, we surprisingly found that the cellular Rev3 level is elevated in mitotic cells, and it is associated with chromatin. Experimental depletion of Rev3 results in elevated CFS expression and chromosomal instability, indicating that Rev3 is required for the late replication of these sites. Unexpectedly, the aforementioned Rev3 activity is independent of Rev7, as the depletion of cellular Rev7 does not cause CFS expression. Furthermore, we found that constitutive depletion of Rev3 in cultured human cells resulted in accumulated genomic instability and eventual arrest of cell division, indicating that Rev3 is required not only for embryonic development but also for cell viability. The significance and implications of this surprising finding are discussed.

MATERIALS AND METHODS

Cell culture

Cultured human cells were maintained in Dulbecco's modified Eagle's medium (Sigma) containing 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25 mM $NaHCO_3$ and 1 × antibiotics/antimycotics (Gibco), with either 10% fetal bovine serum (FBS) (for HCT116) or 10% horse serum (for HeLa). For G_2 cell cycle arrest, cells were treated with 100 ng/ml of nocodazole for 14 h.

Gene knockdown

For transient knockdown, cells were seeded at a density of 10^5/well in a six-well plate and cultured overnight in antibiotic-free media. For each reaction, 50 nM siRNA was mixed with 4 µl of Lipofectamine RNAimax reagent (Invitrogen) as per the manufacturer's guidelines, incubated for 20 min at room temperature and added to the cells. siGenome SMART pool Rev3L siRNA was purchased from Dharmicon (M-006302-01). Previously published siRNA sequences were used to knockdown hRev7 (5′-CCCGGAGCUGAAUCAGUAUTT-3′) (30) and hRev1 (5′-AAGCAUCAAAGCUGGACGACUT T-3′) (31). A non-specific siRNA sequence (D-001810-10, Dharmicon) was used as a control. For stable knockdown, HeLa cells were transduced with shRev1 (sc-38232-v), shRev3 (sc-37790-v) and shRev7 (sc-106795-v) lentiviral particles according to the manufacturer's protocol (Santa Cruz). Scrambled lentiviral particles (sc-108080) were used as a control. After 1 week of selection in the presence of 5 µg/ml puromycin, viable cells were either used for mitotic characterization, or individual cells were seeded to select stable clones.

Quantitative reverse transcriptase–polymerase chain reaction

RNA was extracted with RNEASY® Plus Mini kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad). Quantitative polymerase chain reactions (qPCR) were carried out on an MJ Mini Cycler detection system in the presence of SYBR-Green I dye (Bio-Rad). Primer sequences are available on request. The changes in gene expression were calculated as described in (32).

Immunocytochemistry

Cells seeded onto poly-lysine-coated coverslips and cultured until sub-confluence were washed with ice-cold 1 × phosphate-buffered saline (PBS) before fixing with 4% paraformaldehyde in 1 × PBS for 15 min. Cells were washed with 1 × PBS three times for 5 min each and permeabilized with 0.25% Triton X-100 in 1 × PBS for 10 min at room temperature. After permeabilization, cells were washed three times with 1 × phosphate-buffered saline tween-20 (PBST) and blocked for 30 min in 10% bovine serum albumin (Sigma) or 10% goat serum (Sigma) before an overnight incubation at 4°C with the selected primary antibodies in 5% blocking reagent (1:300 mouse anti-Rev3, produced locally; 1:1000 mouse anti-Rev7, BD-Bioscience; 1:500 rabbit anti-FANCD2, Novus Biologicals). The next day, coverslips were washed three times with 1 × PBST for 10 min each and were incubated with secondary antibodies (Molecular Probes) in 5% blocking reagent (1:2000 Alexa 488 goat anti-mouse; 1:2000 Alexa 546 donkey anti-mouse; and 1:2000 Alexa 546 goat anti-rabbit) for 1 h at room temperature, after which cells were washed three times with 1 × PBST for 15 min each and mounted with 50% glycerol in PBS containing 1.5 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI). Microscopy was performed with an inverted Olympus model IX70, and images were acquired with Image Pro-Plus version 4.1 software and panels compiled using Photoshop version 9. At least three independent experiments were performed for each data set, and secondary antibody alone was included as a negative control.

Cell cycle synchronization and flow cytometry

For cell cycle synchronization, an overnight culture of HCT116 cells at 30–40% confluency was washed twice with 1 × PBS, cultured in serum-free Dulbecco's modified Eagle's medium for 48 h, re-stimulated with 10% FBS media and then collected at indicated time points. For flow cytometry analysis, $\sim 1 \times 10^6$ cells were first washed with PBS and then fixed in 70% ethanol. The cells were stored at 4°C overnight and pelleted at 800 r.p.m. for 5 min the following day. Cells were then resuspended in 1 × PBS and pelleted again at the same speed. The pellet was resuspended in 1 ml propidium iodide solution (1% Triton X-100, 200 µg/ml of

DNase-free RNase A, 20 ng/ml of propidium iodide), filtered through a fine pore mesh and incubated on ice for 30 min before running on a Beckman Coulter Epics XL Flow Cytometer. The data were analysed using FlowJo v9.1.

Western blotting

Whole-cell extract was prepared in a radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris–HCl pH7.4, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulphate (SDS), 2 × protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Sixty to eighty micrograms of protein in 1 × SDS loading buffer was then resolved on a 10% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane using a semi-dry blotting apparatus (Bio-Rad). The membrane was blocked in 5% skimmed milk in 1 × PBS and incubated at 4°C overnight with selected primary antibodies, with β-actin (A-5316, Sigma) at 1:10 000 dilution used as an internal control. Immunoreactivity was detected using horseradish peroxidase-conjugated secondary antibody (1:10 000 goat anti-mouse Upstate 12-349) and developed using Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer NEL104).

Metaphase spreads and fluorescent in-situ hybridization assay

Cells in culture were arrested at G_2 phase by the addition of 0.1 μg/ml colcemid (Sigma) for 3 h. After trypsinization, cells were washed with PBS and treated with hypotonic solution (0.56% KCl) at 37°C for 6 min. The reaction was stopped immediately after the hypotonic treatment by adding three drops of freshly prepared ice-cold methanol/acetic acid (3:1). Cells were centrifuged at 800 r.p.m. for 8 min and were fixed by adding methanol/acetic acid fixative dropwise while vortexing at a low speed. This step was repeated one more time before storing the samples at −20°C for further analysis. To obtain metaphase spreads, cells were dropped on to a pre-cleaned glass slide and dried overnight. Metaphases were then stained with DAPI and visualized under microscope.

For the CFS expression study, pre-labelled CFS probes fragile (FRA)3B (Clone RP11-94D19), FRA16D (Clone RP11-264L1), FRA2G (RP11-527A7) and FRA7H (Clone RP11-36B6) were purchased from The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada. Fluorescent in-situ hybridization was performed according to the manufacturer's instruction (www.tcag.ca). Centromeres were detected with Cy3 human pan centromeric probe (Genetix).

Statistical analysis

For graphs and statistical analysis, Prism v4 software was used. The two-tailed student's *t*-test was used to calculate the significance value. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Expression of both Polζ subunits is elevated in G_2/M cells

We took advantage of a previously characterized anti-Rev3 antibody (33) and monitored the subcellular localization of Rev3 in cultured human cells. As seen in the top panel of Figure 1A, the Rev3 level is much higher in mitotic cells than in interphase cells. To determine whether this enriched signal is indeed Rev3, we performed two sets of experiments. First, treatment of HCT116 cells with siRNA against human Rev3 depleted the putative anti-Rev3 reactivity, including those in mitotic cells (middle panel), and the endogenous Rev3 level was reduced by ∼90% (Figure 1B, lane 3 and Supplementary Figure S1A). Second, HCT116 cells arrested in metaphase after nocodazole treatment displayed enhanced anti-Rev3 staining (Figure 1A, bottom panel), and the cellular Rev3 level is increased by 5-fold compared with cycling cells (Figure 1B, lanes 1 and 2). The aforementioned observations prompted us to ask whether the Rev7 level is also elevated in mitotic cells. Immunocytochemistry (Figure 1C) and WB (Figure 1D) analyses indicate that Rev7 is also accumulated in mitotic cells albeit to a lesser extent. The specificity and efficacy of the anti-Rev7 antibody and siRev7 were assessed in HCT116 cells. Under conditions in which Rev7 was depleted by 93% (Supplementary Figure S1B), the cellular level of Mad2, a homologue of Rev7, was not affected (Figure 1E). On the other hand, depletion of Mad2 by 90% had no effect on the anti-Rev7 antibody reactivity (Supplementary Figure S1C). To determine whether the increased Rev3 and Rev7 levels in mitotic cells are because of transcriptional or post-translational regulation, we performed quantitative reverse transcriptase (qRT)–PCR with synchronized cells. HCT116 cells arrested by serum starvation reached G_2/M phase ∼18 h after release, in which the transcript levels of both *REV3* and *REV7* increased ∼5-fold (Figure 1F), suggesting that the enhanced Rev3 and Rev7 protein levels in mitotic cells are largely because of transcriptional upregulation. Such an unanticipated increase during G_2/M phase has not been reported for any other TLS polymerase in mammalian cells, although a similar phenomenon was reported for Rev1 in yeast cells (34).

Rev3 and Rev7 have distinct subcellular location patters during mitosis

Even though both Rev3 and Rev7 levels are enriched in mitotic cells, we surprisingly noticed different localization patterns in the aforementioned immunocytochemistry experiments: Rev3 seems to be associated with the chromatin (Figure 1A), whereas Rev7 primarily surrounds the chromatin (Figure 1C). To further confirm these observations, we double-stained HCT116 cells with mouse anti-Rev3 and rabbit anti-Rev7 antibodies. The majority of the two proteins primarily localize to different regions during metaphase, anaphase or cytokinesis (Figure 2), a phenomenon further confirmed in U2OS and HeLa cells (data not shown). These observations indicate that under normal growth conditions, the two subunits of Polζ carry out different functions from TLS during G_2/M phase.

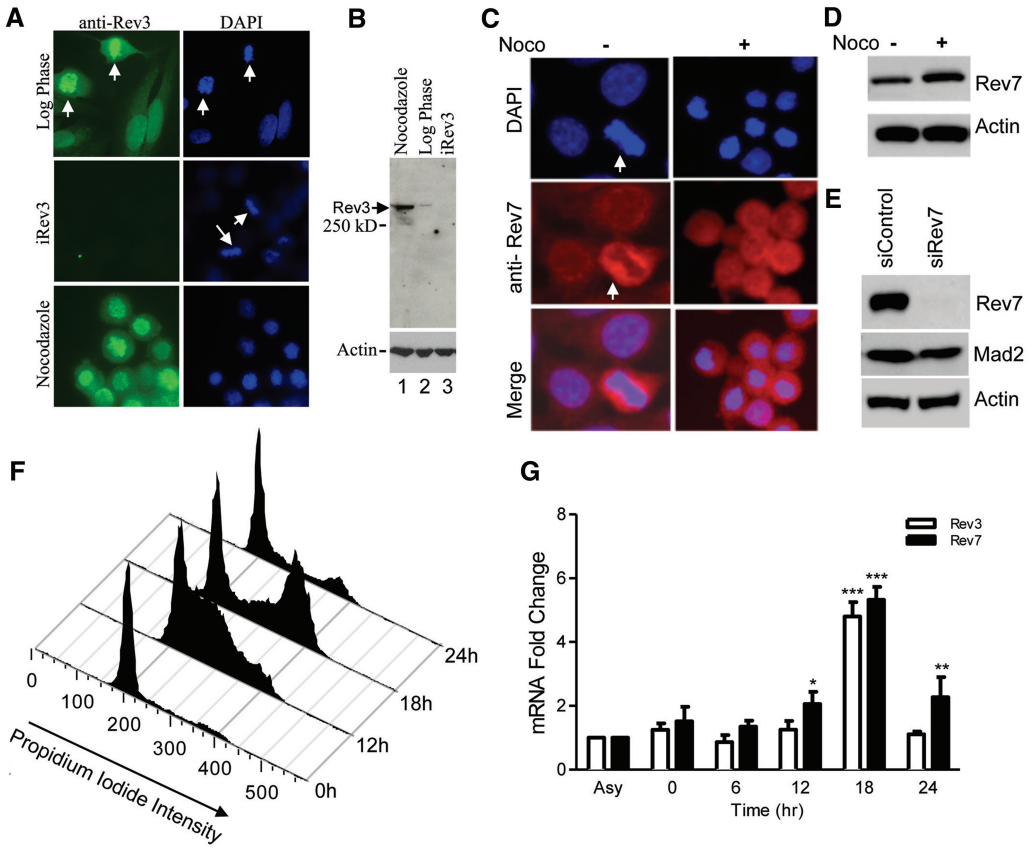

Figure 1. Rev3 and Rev7 expression increases during G_2/M phase in HCT116 cells. (**A**) Cells stained with an anti-hRev3 antibody showing increased chromatin-associated fluorescence in metaphase cells (arrow) as compared with interphase cells (top row). Cells treated with siRNA against Rev3 (iRev3) for >48 h lost Rev3 staining (middle row). Cells arrested at G_2/M-phase by exposure to 100 ng/ml of nocodazole for 12 h showed increased fluorescence in chromatin areas (bottom row). (**B**) Western blot showing higher levels of Rev3 in nocodazole (Noco)-treated cells and abolition on treatment with iRev3. (**C**) Cells stained with an anti-hRev7 antibody either untreated or after treatment with nocodazole in a manner similar to that in (A) showing increased fluorescence in metaphase cells (arrow). (**D**) Western blot showing increased Rev7 levels in nocodazole-treated cells as compared with untreated cells. (**E**) Western blot showing Rev7 knockdown efficiency and specificity 72 h after siRNA transfection. A commercial mouse anti-Rev7 antibody was used for experiments shown in (C–E). (**F**) Cell cycle distribution as determined by flow cytometry after release from 48 h of serum starvation as indicated. (**G**) Relative *REV3* and *REV7* mRNA levels in samples collected from different times after serum starvation as shown in (F) as evaluated through qRT-PCR. Error bars, standard deviation from three samples versus an asynchronized sample (Asy) within the corresponding group. $***P < 0.0005$, $**P < 0.005$ and $*P < 0.05$.

Depletion of TLS polymerases causes distinct mitotic defects

To test a hypothesis that Rev3 and Rev7 have different functions during G_2/M phase, we subjected HCT116 and HeLa cells to transient or stable depletion of Rev3 and Rev7 followed by phenotypic characterization. The depletion efficiency was ~90%, as judged either through qRT-PCR and/or western blotting (Figure 1B and E, and Supplementary Figure S1). After the Rev3 and Rev7 depletion was optimized, two major mitotic defects, namely, anaphase bridges and lagging chromosomes (Figure 3A), were noticed: depletion of Rev3 resulted in a moderate increase in lagging chromosomes (Figure 3B and C), but an up to 4-fold increase in anaphase bridges (Figure 3D and E). In contrast, depletion of Rev7 in the same sets of experiments resulted in nearly 4-fold increase in lagging chromosomes (Figure 3B and C), with little effect on anaphase bridges (Figure 3D and E). A similar lagging chromosome defect was reported for Rev7 recently (36,37), vindicating the idea that Rev7 has more to do with proper segregation and/or alignment of chromosome at the metaphase plate.

In the aforementioned experiments, we also included Rev1 knockdown as a reference of TLS, as it has been shown that the recruitment of both Rev3 (33) and Rev7 (P.A. and W.X., unpublished data) to ultraviolet (UV)-induced DNA damage sites is dependent on Rev1 but independent of Polη. In both experimental systems, depletion of Rev1 moderately increased lagging chromosome frequencies but had no effect on anaphase bridges (Figure 3). To ask whether increased anaphase bridges are because of dicentric chromosomes, we performed FISH on metaphase cells using a centromeric probe. Even though depletion of Rev3 resulted in a marginal increase in dicentric chromosomes, it was not significantly higher than any other groups studied (Supplementary Figure S2), which excludes chromosome end-fusion as a major cause of anaphase bridge formation in Rev3-depleted cells. These observations support a notion that Rev3 plays a unique role in chromosome separation independent of its role(s) in TLS.

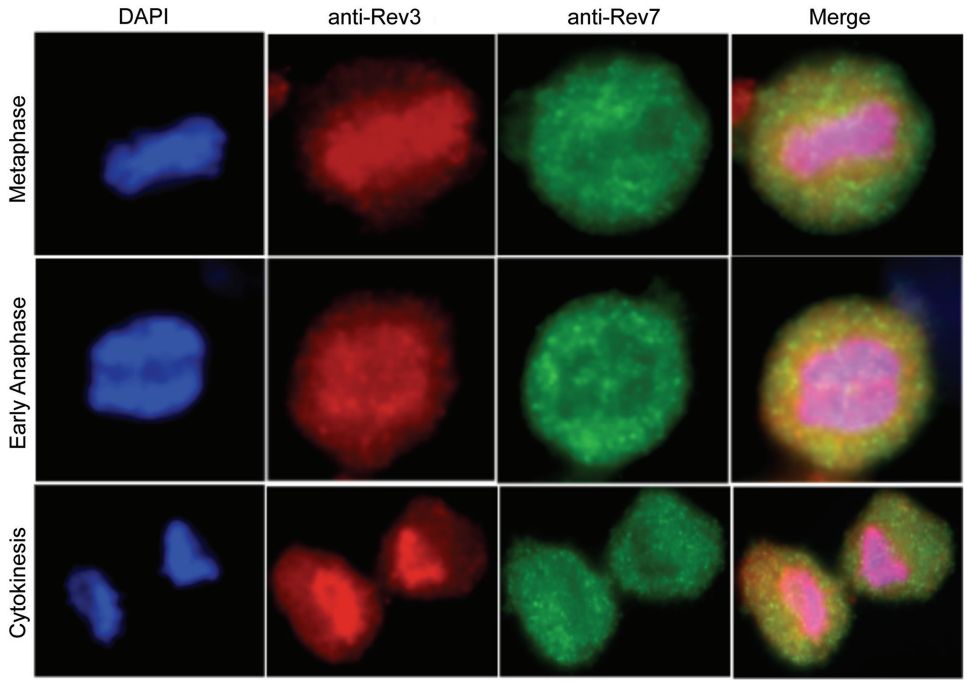

Figure 2. Rev3 and Rev7 subcellular localization in HCT116 cells. Representative images from cells stained with the mouse anti-hRev3 (Red) and a rabbit anti-hRev7 (Green, received from Dr V. Maher, University of Michigan) antibodies (35) showing distinct subcellular distribution patterns in G_2/M cells. Cell cycle stages are indicated on the left panel. DAPI stain represents DNA.

Depletion of Rev3, but not Rev7, causes mitotic chromosome breaks and CFS expression

To ask what type(s) of chromosomal instability leads to increased anaphase bridges in Rev3-depleted cells, metaphase spreads were examined from cells collected at different time points after target protein depletion. Loss of Rev3 in HCT116 led to five times more chromatid breaks/gaps ($P = 0.003$) than control cells, which was in sharp contrast to Rev1- or Rev7-depleted cells (Figure 4). Highly consistent results were also obtained in HeLa cells (Supplementary Figure S3). During the preparation of this manuscript, Lange *et al.* (38) reported a similar phenotype in mouse fibroblasts using a conditional *Rev3* knockout approach, confirming that the increase in chromosomal breaks is because of loss of Rev3.

To further understand the cause of such chromosomal instability, we relied on the fact that the observed breaks/gaps resemble those found in cells undergoing replication stress (10), and wondered whether a similar effect is involved in Rev3-depleted cells as well. To answer this question, we first treated Rev1-, Rev3- and Rev7-depleted HCT116 cells with a low dose of phidicolin (APH) to induce partial replication stress and then used CFS-specific DNA probes to locate the breaks. APH, a DNA polymerase α and δ inhibitor, is known to induce CFS expression when used at low concentrations without affecting cell cycle progression (39). As expected, Rev3-depleted cells showed significantly more breaks/gaps when compared with control in the same treatment group (Figure 5A), suggesting that Rev3 plays a role in the maintenance of CFSs. This observation is similar to what has been reported for other genes involved in CFS stability, such as ataxia telangiectasia and Rad3 related (ATR) and FANCD2 (10,11). We also observed higher shattered metaphase defect in Rev3-depleted cells treated with 0.2 μM APH than any other studied group (Figure 5B and C). To confirm that Rev3-depletion indeed causes CFS expression, we took advantage of previously well-characterized CFS probes (FRA2G, FRA3B, FRA7H and FRA16D) based on their high expression in human cells (4). As shown in Figure 5D and E, suppression of Rev3 led to strong spontaneous CFS expression at all sites examined.

Depletion of Rev3 induces mitotic FANCD2 and γH2AX focus formation

The relationship between some Fanconi anaemia (FA) pathway genes especially *FANCD2* and fragile site stability has long been known (11,40,41), but it was only until recently that FANCD2 was reported to form foci at the sites representing unresolved replication intermediates that also happen to generate anaphase bridges (42). Furthermore, these foci were found primarily at the fragile sites on APH treatment, suggesting that FANCD2 marks regions undergoing replication stress. As our data suggest that Rev3 depletion causes fragile site expression in a manner similar to that caused by replication stress, we wondered whether Rev3 inactivation also induces FANCD2 focus formation. Compared with control cells, twice as many Rev3-depleted mitotic cells contain more than two FANCD2 foci, whereas under the same experimental conditions, Rev7 depletion has no effect on FANCD2 focus formation (Figure 6A and B). In a similar experiment when cells were treated with 0.1 μM APH for 24 h after the gene knockdown, Rev3-depleted

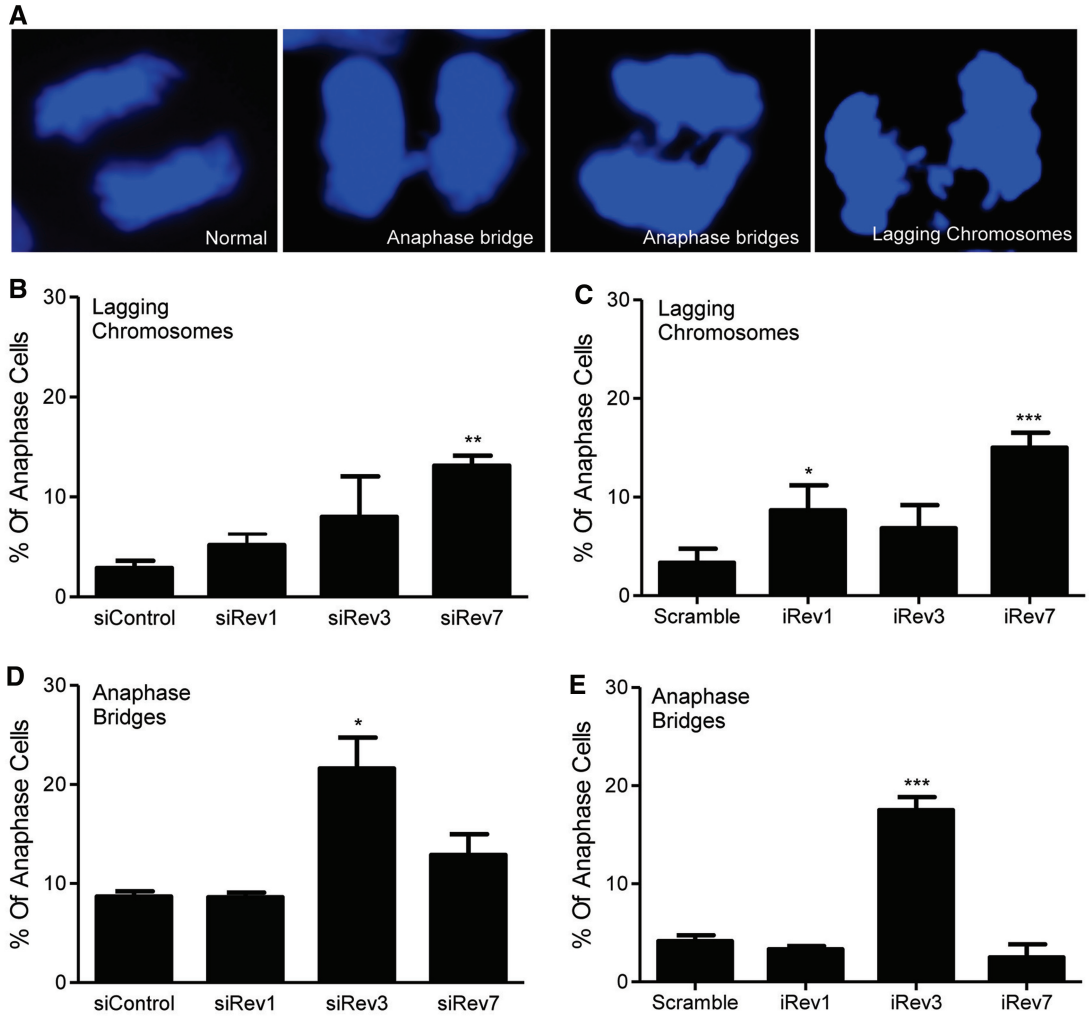

Figure 3. Rev3 and Rev7 suppression causes distinct types of genomic instability. (**A**) Representative anaphase HCT116 cells with DAPI staining showing bridges and lagging chromosomes. (**B**) Quantitative analysis of lagging chromosomes in cells treated for 48 h either with non-specific siRNA or siRNA against Rev1, Rev3 or Rev7. (**C**) Quantitative analysis of lagging chromosomes in HeLa cells transduced either with scrambled lentivirus or lentivirus expressing siRNA against Rev1, Rev3 or Rev7. (**D**) Quantitative analysis of anaphase bridges in same cells as in (B) but treated with siRNA for 72 h. (**E**) Quantitative analysis of anaphase bridges from same experiment as described in (C). (B–E) error bars represent standard deviations from three independent experiments with >100 anaphase cells for each experiment. $*P < 0.05$, $**P < 0.005$ and $***P < 0.0005$ versus control.

cells exhibited more metaphase cells with ≥10 FANCD2 foci than siRev7- or siControl-treated cells (Supplementary Figure S4A and B), suggesting that loss of Rev3 further perturbs DNA replication in cells challenged with replication inhibitors. Next, by using γH2AX focus formation as a marker, we asked whether the metaphase-specific FANCD2 foci simply represent double-strand breaks (DSBs). As shown in Figure 6C and D, 40% of the metaphase FANCD2 foci either showed complete overlap or close proximity to γH2AX, whereas the remaining 60% were localized independently. This is consistent with a previous report showing that only a portion of the FANCD2 foci co-localize with γH2AX foci (42).

Rev3 is essential for human cell proliferation

As we did not observe any immediate cell death on Rev3-depletion, we wondered whether long-term depletion could impact cell survival. To this end, we generated numerous siRev3-containing lentiviral-transfected HeLa cell lineages. After selecting cells for 2–3 weeks with puromycin, ~25 clones were expanded and examined, among which 4 clones (C4, C7, C8 and C19) with >50% reduction and 2 (C21 and C22) with almost no change in Rev3 levels (Figure 7A) were further examined. As shown in Figure 7B and C, depletion of Rev3 led to increased nuclear defects, including micronucleation, multinucleation, nuclear bridges, abnormal nuclei with poor growth and cell death in all clones except C21 and C22. The overall level of nuclear defects seems to be closely correlated with the efficacy of Rev3-depletion (compare Figure 7A and B). Furthermore, we were unable to obtain stable Rev3-depletion cell lines, as most transfectants only formed micro-colonies, and those that formed colonies regained Rev3 expression. In contrast, long-term depletion of Rev1 or Rev7 by the same approach did not cause the

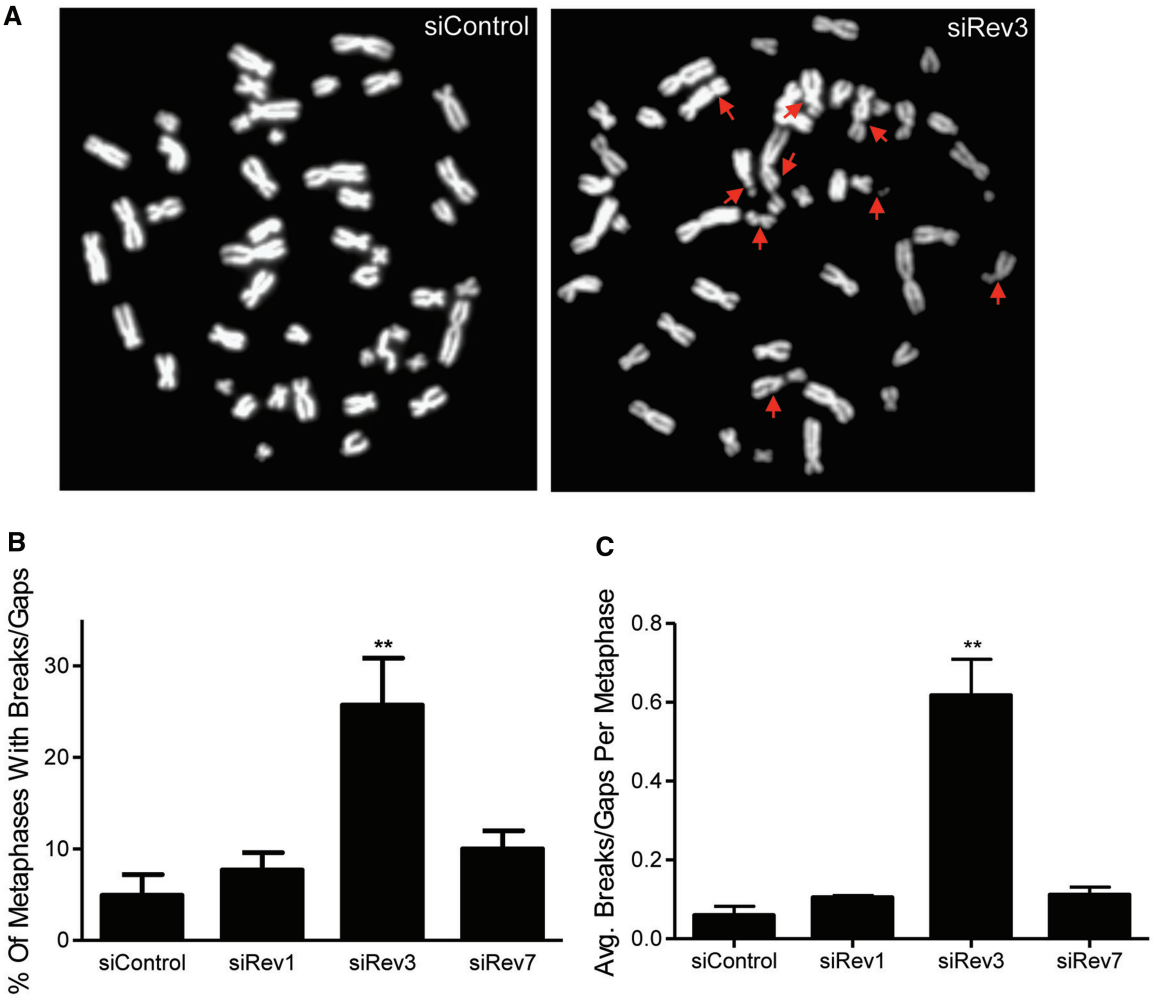

Figure 4. Loss of Rev3 causes DSBs in HCT116 cells. (**A**) Representative images of metaphase spreads showing breaks/gaps (arrow) 72 h after siRev3 treatment. (**B**) Percentage of metaphase cells containing breaks/gaps after the indicated siRNA treatment. (**C**) Quantitative analysis of average breaks/gaps per metaphase cell after siRNA treatment. Error bars represent standard deviations from three independent experiments with >50 metaphases for each experiment. $^{**}P < 0.005$ versus control.

accumulation of the aforementioned nuclear defects or the formation of micro-colonies (data not shown). Together with our previous observations (43) using a different cell line and stable siRNA targeting strategy, we conclude that Rev3 plays an essential role in cell proliferation.

DISCUSSION

Advances in the mechanism of CFS instability have provided new insights into not only their possible cause of expression but also DNA replication in general (1,2). One recent study is of particular interest, as it was demonstrated for the first time that CFS instability is probably because of the long distance a replication fork has to travel while there are few initiation events in the CFS core region spanning several hundred kilobases (3). However, it is also clear that many proteins, including replication checkpoint sensors, helicase and topoisomerase, also play important roles in the maintenance of CFSs (5,6). The role of replicative polymerases in CFS stability is only revealed when cells are exposed to sub-lethal doses of polymerase inhibitors, such as APH. Here, we report that the Rev3 catalytic subunit of Polζ is required for the maintenance of CFS stability, and that this function is probably independent of its regulatory subunit Rev7. It also suggests that Rev7 only serves to recruit Rev3 to the DNA damage site during TLS, and it may not be the functional partner of Rev3 during CFS replication.

Three lines of evidence support the involvement of Rev3 in the maintenance of CFS stability. First, the Rev3 level is elevated and enriched on the condensed chromatin in G_2/M-phase cells. Interestingly, replication of the CFS core region also occurs during G_2/M phase (1–3), suggesting a possible correlation between elevated Rev3 and CFS replication. This is important, as TLS polymerases are believed to function during S and/or G_2 phase, and no such increase has been reported for other TLS polymerases in eukaryotic cells, except for Rev1 in yeast cells (34). As Rev3 and Rev7 have distinct subcellular localization in mitotic cells and mitotic phenotypes on their depletion, it is highly unlikely that this is a TLS-related function.

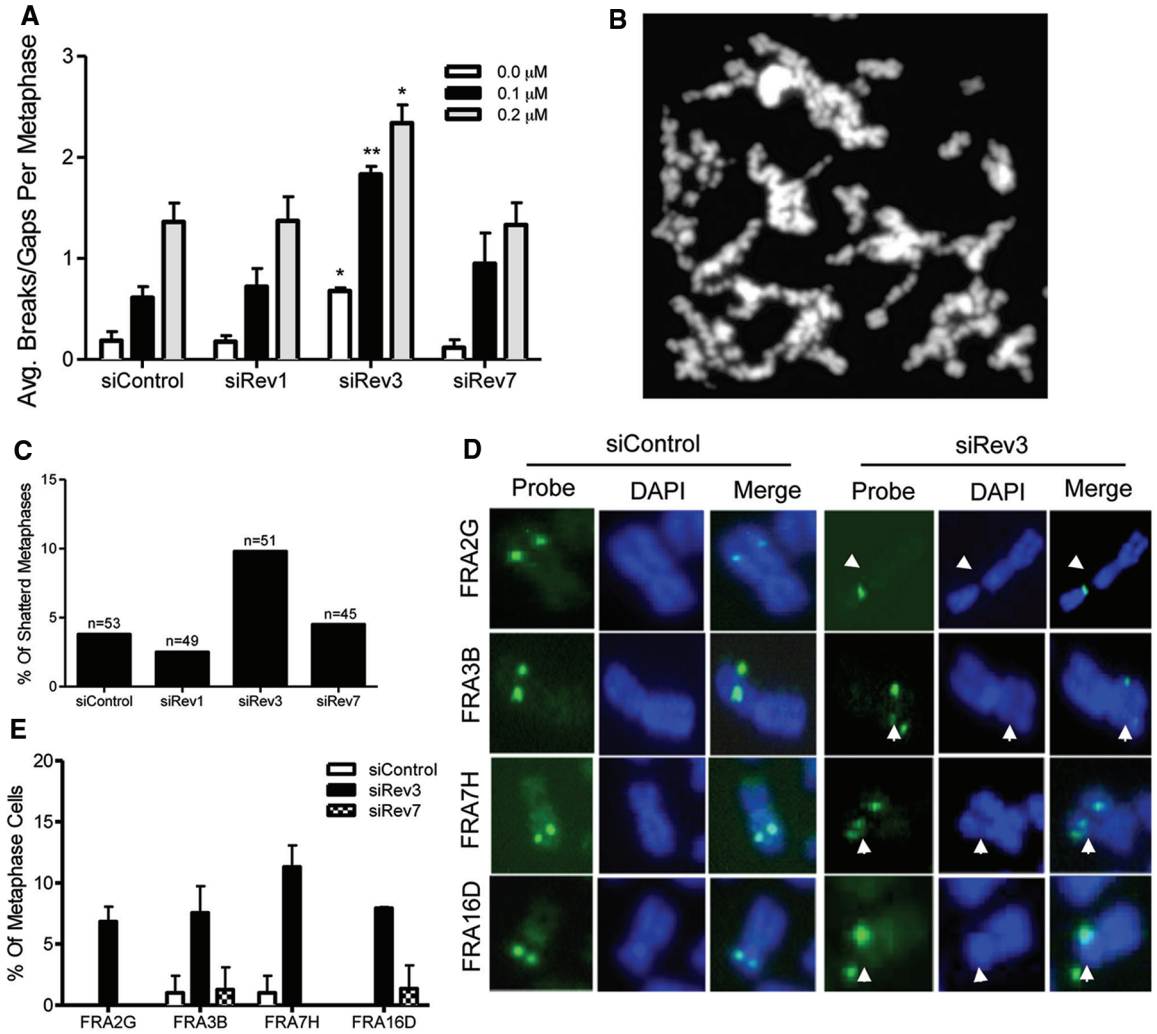

Figure 5. Depletion of Rev3 in HCT116 cells causes fragile site expression and increased genomic instability. (**A**) Quantitative analysis of average breaks/gaps per metaphase after siRNA-treated cells were exposed to APH. Cells were incubated with the indicated siRNA for 45 h before a 24-h APH treatment followed by a 3-h colcemid treatment before being fixed and analysed. Shattered metaphases were not included in this analysis. *P*-value was calculated within each treatment group in comparison with the control siRNA treatment. $^{**}P < 0.005$, $^{*}P < 0.05$. (**B**) Representative metaphase spread from Rev3-depleted cells followed by 0.2 μM APH treatment showing the shattered chromosome phenotype. (**C**) Percentage of metaphases with shattered chromosome phenotype after siRNA and 0.2 μM APH treatment. (**D**) Representative chromosomes showing spontaneous breaks (arrow heads) at their respective fragile sites on Rev3 depletion, detected through FISH using indicated fluorescent fragile site probes. (**E**) Quantitative analysis of data as presented in (D). All error bars in this figure represent two independent experiments with >50 metaphases analysed for each experiment.

Second, depletion of Rev3 leads to increased genomic instability. The high prevalence of anaphase bridges in Rev3-depleted cells that did not represent dicentric chromosomes is in agreement with a previous report (44) that cells undergoing replication stress have a high frequency of anaphase bridges, especially ultrafine bridges. Furthermore, the increased number of FANCD2-positive anaphase bridges is also in agreement with a previous report (42), suggesting that these anaphase bridges are the consequence of replication stress, most probably representing underreplicated intermediates. The high number of breaks/gaps per metaphase in Rev3-depleted cells as compared with other treated groups suggests that loss of Rev3 causes genomic instability similar to cells undergoing replication stress (7). This is unlikely because of its role in the repair of DSBs, as we did not find any significant difference in the number of tri- or tetra-radial chromosomes between different treated groups (Supplementary Figure S4), and, importantly, the above repair also involves Rev7 and Rev1 (27). The increased breaks/gaps in Rev3-depleted cells agree with a recent report in mouse fibroblasts (38), suggesting that Rev3 has a similar function in human cells as well, although the mechanism behind such instability has not been addressed. The selective use of low doses of DNA replication inhibitor APH, which caused significantly higher breaks/gaps per metaphase in Rev3-depleted cells, supports a notion that Rev3 has a role in CFS replication. The third and direct line of evidence is the data from the FISH assay using four different CFS probes. All fragile sites examined showed

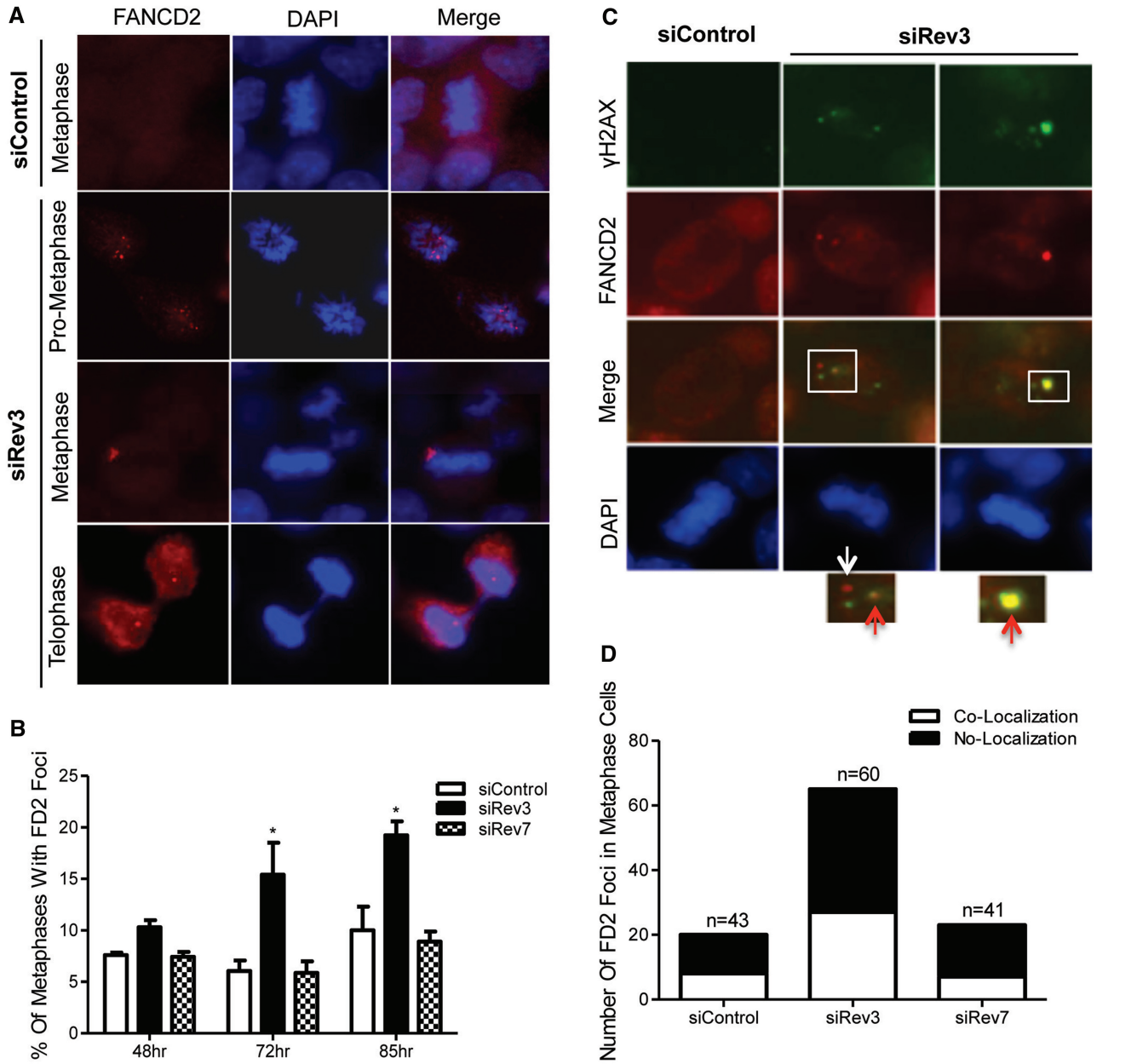

Figure 6. Depletion of Rev3 leads to FANCD2 focus formation in HCT116 metaphase cells. (**A**) Representative images of cells depleted of Rev3 for 72 h and stained with an anti-FANCD2 (FD2) antibody showing the FANCD2 focus formation. A metaphase with no focus (top row), multiple foci on pro-metaphase (second row), cluster of foci on metaphase (third row) and two distinct foci on each side of the anaphase bridge (bottom row). (**B**) Quantitative measurement of metaphases/pro-metaphases with more than two foci per cell after 48, 72 and 85 h of treatment with siRNA. Error bars represent two experiments with >50 metaphase cells per experiment. $^*P < 0.05$. (**C**) Representative images showing FANCD2 and γ-H2AX focus formation and their co-localization in metaphase cells. Cells were treated with gene-specific siRNA for 72 h and then stained with rabbit anti-FANCD2 and mouse anti-γH2AX antibodies simultaneously. Two distinct types of metaphase-specific FANCD2 foci were observed; one is co-localized with γH2AX foci (red arrow, enlarged box) and another does not correlate with γH2AX (white arrow, enlarged box). Boxed regions are enlarged at the bottom. (**D**) Proportion of metaphase FANCD2 foci with or without γH2AX focus co-localization.

significantly elevated expression in Rev3-depleted cells, which allowed us to conclude that Rev3 is required for CFS stability.

Our current knowledge about the maintenance of CFSs in mammals suggests that it is a complex process and involves many factors, including DNA-replicative polymerases. It is possible that owing to the complex nucleotide architecture of these late-replicating regions, replicative polymerases alone may not be efficient enough to complete their replication; hence, Rev3 may play a crucial role in CFS replication. Based on observations reported here, we envision that Rev3 is recruited to CFSs by an unknown factor in a manner similar to its recruitment to the DNA damage site and contributes to CFS replication along with replicative polymerases. It would be of great interest to identify such unknown factor(s) and determine whether the Rev3 polymerase activity is essential for its CFS maintenance function.

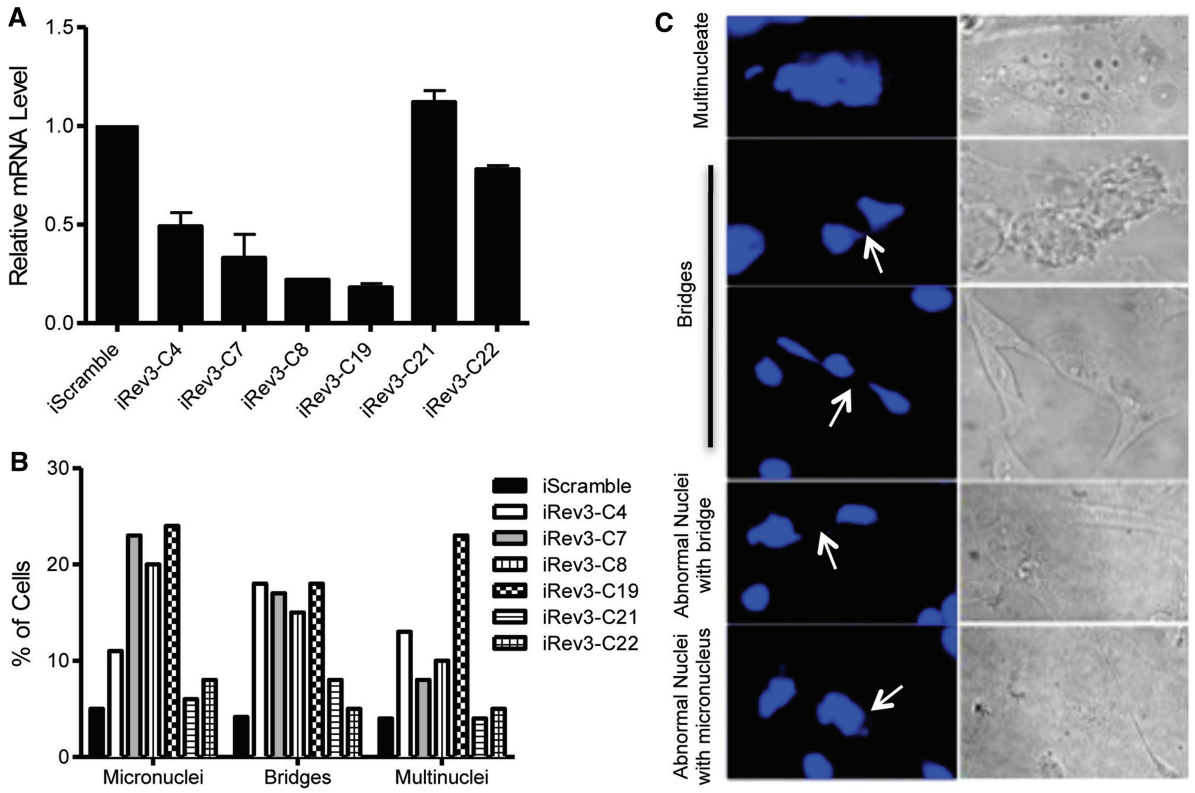

Figure 7. Prolonged depletion of Rev3 in HeLa cells causes multiple nuclear defects leading to cell death. (**A**) Relative *REV3* mRNA levels of individual clones after 3 weeks of transduction by lentivirus expressing siRNA against *REV3*. (**B**) Nuclear defect frequency in selected clones. For each clone >100 cells were counted. (**C**) Representative images showing various forms of chromosomal abnormalities as indicated in (B).

It should be noticed that because of experimental limitation, we cannot formally exclude a possibility that the residual amount of Rev7 after depletion is sufficient to support the Polζ activity in mitotic cells for the maintenance of CFS stability. Interestingly, it was recently reported that yeast Polζ forms a complex with Polδ subunits Pol31 and Pol32 (45–47), although it remains unclear whether such a complex exists in mammalian cells as well, and, if yes, whether it is limited to polymerase switch during TLS. It is also noticed that the budding yeast *REV3* gene is not essential for cell survival or cell cycle progression, although putative fragile sites, or replication slow zones, have been identified in the yeast genome as well (48–50). Comparison of predicted yeast and mammalian Rev3 proteins reveals a large exon that is unique to the mammalian Rev3, which will be subjected to future investigations for its role(s) in the maintenance of mitotic genomic stability.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–5.

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