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FANCJ is essential to maintain microsatellite structure genome-wide during replication stress

Joanna Barthelemy[1], Helmut Hanenberg[2,3] and Michael Leffak[1,*]

[1]Department of Biochemistry and Molecular Biology, Boonshoft School of Medicine, Wright State University, Dayton, OH 45435, USA, [2]Department of Pediatrics III, University Children's Hospital Essen, University of Duisburg-Essen, 45122 Essen, Germany and [3]Department of Otorhinolaryngology & Head/Neck Surgery, Heinrich Heine University, 40225 Duesseldorf, Germany

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ABSTRACT

Microsatellite DNAs that form non-B structures are implicated in replication fork stalling, DNA double strand breaks (DSBs) and human disease. Fanconi anemia (FA) is an inherited disorder in which mutations in at least nineteen genes are responsible for the phenotypes of genome instability and cancer predisposition. FA pathway proteins are active in the resolution of non-B DNA structures including interstrand crosslinks, G quadruplexes and DNA triplexes. In FANCJ helicase depleted cells, we show that hydroxyurea or aphidicolin treatment leads to loss of microsatellite polymerase chain reaction signals and to chromosome recombination at an ectopic hairpin forming CTG/CAG repeat in the HeLa genome. Moreover, diverse endogenous microsatellite signals were also lost upon replication stress after FANCJ depletion, and in FANCJ null patient cells. The phenotype of microsatellite signal instability is specific for FANCJ apart from the intact FA pathway, and is consistent with DSBs at microsatellites genome-wide in FANCJ depleted cells following replication stress.

INTRODUCTION

Fanconi anemia (FA) is a rare, recessive disorder usually diagnosed in childhood and clinically characterized by congenital anomalies, progressive bone marrow failure and a high propensity to develop malignancies, predominantly acute myeloid leukemia and squamous cell carcinomas (1,2). Genetically, loss-of-function mutations in at least 19 FA genes, which define the FA complementation groups, lead to inefficient repair of DNA interstrand crosslinks (ICLs) and other cellular defects (3).

Based on the chronology of their action in the FA pathway and their biochemical characteristics, the FA proteins can be grouped into sensors of ICLs, stabilizers of stalled replication forks, mediators of ICL excision and effectors of fork restart by homologous recombination (HR). The convergence of DNA replication forks on an ICL generates ssDNA that activates the Ataxia telangiectasia and Rad3 related (ATR) checkpoint kinase to phosphorylate the FANCI/D2 heterodimer. Mono-ubiquitination of phosphorylated FANCI/D2 by the FA core complex (FANCA, -B, -C, -E, -F, -G, -L and –M) promotes binding at the ICL and recruitment of the FANCP/SLX4 scaffold and its associated nucleases. 5′ and 3′ incisions unhook the ICL from the lagging strand template to produce a DNA double strand break (DSB) that can be resected by MRN-CtIP, EXO1 and DNA2 to generate 3′ ssDNA ends that re-establish the replication fork by FANCJ (BRIP1, BACH1)/FANCS (BRCA1)/FANCD1 (BRCA2)/RAD51-mediated HR (4,5).

The FA proteins are also required for normal DNA replication and the response to replication stress (6–18). Thus, several of the FA proteins act in other DNA repair pathways including nucleotide excision repair, mismatch repair and HR. An example is the FANCJ helicase, which binds to BRCA1, MRN, TOPBP1, RPA, MLH1 and BLM (19,20). Mutations that block these interactions reduce cell survival under replication stress, as do mutations that eliminate FANCJ helicase and protein displacement activities (8,18). Knockdown of FANCJ also inhibits HR (21).

FANCJ patient cells are hypersensitive to ICL reagents such as mitomycin C (MMC) and other inhibitors of DNA polymerases (21–23), supporting a direct role for FANCJ in DNA replication. Human FANCJ is able to unwind G quadruplex (G4) and DNA triplex structures (24–26), while mutation of the *Caenorhabditis elegans* FANCJ homolog dog-1 results in loss of DNA sequences flanking long runs of guanines (27,28). G4s have been observed to form in human cells (29) and human cells in which mutant FANCJ protein is deficient in G4 unwinding accumulate deletions at or near G4 consensus sequences (25). With an estimated 300 000 G4 consensus repeats per human genome (30), FANCJ depletion is a potential cause of widespread chromosome instability.

*To whom correspondence should be addressed. Tel: +1 937 775 3125; Fax: +1 937 775 3730; Email: michael.leffak@wright.edu

Human microsatellite sequences that share the tendency to form non-B DNA structures are proposed hotspots for chromosome breakage and translocations (reviewed in (31)). Thus, inverted Alu repeats prone to hairpin formation and a late replicating AT-rich common fragile site fragment comprising 34 AT dinucleotide repeats which is predicted to adopt a non-B DNA secondary structure, cause DSBs and inhibit DNA repair *in vivo* (32). In murine stimulated lymphocytes after acute treatment with 10 mM hydroxyurea (HU), early replicating fragile sites (ERFS) were defined by co-localization of the single strand binding protein RPA and the HR proteins BRCA1 and SMC5 to large (>60 kb) fragile regions identified at the resolution of FISH. ERFS were enriched for LINE and SINE sequences but not for simple sequence repeats. In contrast, we have shown that short CTG/CAG repeats form hairpins in vivo (33) and that replication through CTG/CAG repeats is blocked relative to replication in the opposite direction from the same origin in human cells (33,34). Expanded CTG/CAG repeat sequences are also sites of replication fork slowing and chromosome breakage in *Saccharomyces cerevisiae* (35). Moreover, an asymmetric purine/pyrimidine (pu/py) repeat that forms triplex DNA and G4 structures blocks replication and causes a constitutive DNA damage response *in vivo* (36).

To test the hypothesis that FANCJ is involved in the replication of non-B DNA structures, we designed HeLa cells with an integrated copy of an expanded $(CTG/CAG)_{102}$ microsatellite repeat that forms DNA hairpins on the leading and lagging arms of a replication fork *in vivo* (33). Using small pool polymerase chain reaction (spPCR) with primers close to the $(CTG/CAG)_{102}$ trinucleotide tract we found that the ectopic microsatellite could not be amplified by spPCR in FANCJ depleted HeLa cells after treatment with low dose aphidicolin (APH) or HU. These results suggest that replication stress induces DSBs or other changes inimical to PCR at the $(CTG/CAG)_{102}$ repeats. Consistent with replication-dependent microsatellite DSBs, inverse PCR and DNA sequence analysis showed ectopic site recombination to other non-B DNA repeat sites associated with genome aberrations in several forms of cancer.

Surprisingly, microsatellites at multiple endogenous loci across the genome also showed PCR signal loss in FANCJ depleted cells under replication stress. These results were recapitulated in FANCJ null patient fibroblasts, but not in cells with defects in other proteins of the FA pathway. These results are consistent with the view that FANCJ is required for the stabilization of numerous kinds of structure-prone repetitive sequences throughout the human genome during DNA replication and that depletion of FANCJ results in chromosome double strand breaks near these microsatellite repeats.

Since this phenotype is not observed in FA cells with loss-of-function mutations in other FA genes, the maintenance of microsatellites is independent of the classical FA repair pathway. These results indicate that FANCJ is required for accurate replication of a variety of repeated sequences across the genome that can adopt non-canonical DNA structures. Recombination at repetitive sequences during replication stress suggest that microsatellite DNAs are sources of the chromosomal aberrations found in FANCJ patients and may contribute to oncogenesis and developmental abnormalities.

MATERIALS AND METHODS

Cell culture

$(CTG/CAG)_{102}$ cell lines were derived from transfected HeLa cells containing a single FLP recombinase target site as described previously (37). The sequence of the control, non-repetitive ectopic site DNA (Supplementary Figure S1) is available from the authors. Primary FANCJ null (FA752), FANCC (FA858), FANCQ (FA844) and FANCI (FA705) skin fibroblasts were kindly obtained from Detlev Schindler (Human Genetics, University of Wuerzburg, Germany). Cells were immortalized as previously described (3,38). FANCJ null cells (FA752) were complemented with wild-type FANCJ (FA752 + FANCJ WT) or transduced with a *neo* control vector as described (38,39), RA3087 (FANCA), RA3331 (FANCP), RA2645 (FANCD2), RA3100 (FANCL), RA3226 (FANCD1/BRCA2) and RA2374 ($FANCJ^{R798X}$) FA patient immortalized fibroblast cells were provided by Agata Smogorzewska (Rockefeller University). Normal fibroblasts (GM08402) were obtained from Coriell Cell Repositories. All cells were grown in Dulbeco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

siRNA treatment

$(CTG/CAG)_{102}$ cells (60% confluent) were reverse transfected with siPort NeoFX reagent (Invitrogen 4510M) and 100 nM (final concentration) of small interfering RNA (siRNA) targeting FANCJ/BRIP1 (siGENOME M-010587) FANCM (siGENOME M-021955), or FANCD2 (siGENOME M-016376) (Dharmacon Research, Inc.) in 6-well plates every 48 h for a total of five transfections in the presence of 0.2 μM APH or 0.3 mM HU. Cells were harvested 48 h post-transfection. Control experiments were carried out using AllStars negative non-targeting siRNA (Qiagen SI03650318). Fibroblast cell lines were treated with 0.5 μM APH or 0.3 mM HU for 10 days. Control, untreated counterparts were plated simultaneously.

PCR

Small pool PCR (spPCR) used 50 pg of genomic DNA per reaction. Each spPCR occurred in the presence of HotStart Taq polymerase MasterMix (Qiagen). Amplification conditions were 95° (15 min); 35 cycles of 94° (1 min), 54° (45 s), 72° (1 min); final 72° (7 min). PCR products were resolved on 8% polyacrylamide gels stained with GelRed (Biotium). Images were obtained on a Fuji LAS-3000. See Supplementary Table S1 for PCR primers used in this work.

Inverse PCR (iPCR) samples were derived from CTG_{102} cell DNA that had been treated with siFANCJ and APH (0.2 μM). DNA (2.5 ug) was digested with an excess of MSEI (New England BioLabs, Inc.) for 1 h at 37° in a 50 μl total volume reaction. After a 20-min heat inactivation at 65°, 10 μl of the reaction was diluted to 1 ng/ul DNA and used for ligation in the presence of T4 DNA ligase (New England BioLabs, Inc.) for 1 h at 16°. The ligation reaction products were cleaned using the MinElute

Reaction Cleanup Kit (Qiagen). Amplification used inverse PCR primers (CGCTCAGTGGAACGAAAACT, AGAC CCCGTAGAAAAGATCAAAGGA) and HotStart Taq polymerase Mastermix (15 min at 95°; 35 cycles of 1 min at 94°, 45 s at 58°, 1 min at 72°; final 7 min at 72°). iPCR products were cleaned with a Cycle Pure Kit (Omega D6492) and used for next generation sequencing.

Western blotting

Whole cell lysates from treated or untreated HeLa cells or fibroblast cells were prepared. After sodium dodecyl sulphate-polyacrylamide gel electrophoresis, membranes were probed using a 1:500 dilution of primary antibody (rabbit FANCJ/BRIP1 (Abcam ab151509), mouse FANCM provided by Lei Li (University of Texas MD Anderson Cancer Center), mouse FANCD2 (Santa Cruz Biotechnology, Inc. SC-20022) or mouse β-actin (Sigma-Aldrich A5441)). After incubation with secondary antibody, membranes were imaged on a Fuji LAS-3000 using ImageReader.

Aphidicolin sensitivity

HeLa cells were transfected with FANCJ siRNA or control siRNA by reverse transfection. Continuous APH (0.2 μM) treatment began 24 h after the first transfection (0 h). Fibroblast cells were treated continuously with APH (0.5 μM) for 96 h. Viable cells were counted by trypan blue (Sigma-Aldrich) exclusion.

Next generation sequencing

DNA sequencing was performed by the Case Western Reserve University Genomics Core facility. The Illumina Nextera XT kit was used for library construction. This kit uses an engineered transposome to tagment genomic DNA, and eliminates ligase reactions, resulting in minimal 'chimeras'. Library quality was verified by Agilent 2100 Bioanalyzer and quantified by qPCR (KAPA Biosystems) before analysis on the MiSeq instrument. Our analysis focused on the ca. 28 000 reads that contained breakpoint junction sequences out of ca. 26 million total reads. There was also a pool of 999 low abundance reads, of which 990 were present as 1 read, and 9 were present as 2 reads. These reads were judged to be *in vitro* 'chimeras' as they appeared to occur randomly over the genome, and were at least 40- to 10 000-fold less abundant than junction reads attributed to recombination.

RESULTS

FANCJ is required to maintain ectopic site CTG/CAG trinucleotide repeats

The FANCJ helicase interacts with multiple proteins implicated in cellular responses to non-canonical forms of DNA and replication stress. Among the sequences that slow replication forks in human cells are CTG/CAG hairpins, and asymmetric purine/pyrimidine (pu/py) tracts able to form DNA triplexes and G4s (33,34,36). Since FANCJ unwinds non-B DNA structures that inhibit DNA replication, we first tested the effect of FANCJ depletion on the stability of an expanded $(CTG/CAG)_{102}$ microsatellite that forms leading and lagging strand hairpins *in vivo* (33).

Yeast FLP recombinase was used to create a pair of clonal HeLa cell lines (CTG_{102}, CAG_{102}) that each contain a single ectopic integrant of the human 2.4 kb c-myc core replication origin flanked by a $(CTG/CAG)_{102}$ microsatellite tract (Figure 1A) (33,34). When replicated from the flanking c-myc origin, CTG_{102} cells or CAG_{102} cells, respectively, have the CTG repeat or the CAG repeat in the lagging strand template. FANCJ was efficiently knocked down using siRNA (Figure 1B and D) in the absence or presence of a low dose of APH (0.2 μM APH) that does not acutely activate the replication checkpoint but has been previously used to slow DNA polymerization and reveal chromosome fragile sites (40–42). To assess the fluctuation between cells in the population and detect microsatellite instability (expansions, contractions, DSBs) genomic DNA was amplified by spPCR using primers within 85 bp 5′ and 3′ to the ectopic $(CTG/CAG)_{102}$ repeats, in the presence of an internal PCR control primer set (33,34,43). In contrast to repeat expansions and contractions observed after repeated cycles of replication inhibition and restart (33,34,43), we observed that the spPCR signal of the ectopic $(CTG/CAG)_{102}$ repeats was dramatically decreased in DNA from cells depleted of FANCJ and treated continuously with APH (Figure 1C), and this effect could be reproducibly detected by end-point PCR. These results are consistent with double strand DNA breaks occurring within 85 bp of the $(CTG/CAG)_{102}$ tract that separates the flanking PCR primers, although other explanations are formally possible (see 'Discussion' section). In contrast, spPCR of sequences 1–3 kb from the ectopic site did not reveal loss of flanking DNA (Supplementary Figure S1A–D). In addition, the population doubling time of the FANCJ siRNA treated cells was hypersensitive to APH (Supplementary Figure S1E).

To confirm that the ectopic site was not inducing PCR signal loss of an otherwise stable CTG/CAG microsatellite, we created a cell line with a non-repetitive control sequence flanking the c-myc origin integrated at the same ectopic site. Importantly, FANCJ knockdown in the presence of 0.2 uM APH did not induce loss of the ∼2.1 kb fragment spanning the ectopic site non-repeat sequence (Supplementary Figure S1G).

To test whether the loss of ectopic microsatellite signals was unique to APH treatment, CAG_{102} and CTG_{102} cells transfected with FANCJ siRNA were alternatively treated with the ribonucleotide reductase inhibitor HU that depletes dNTP pools and inhibits replication. At low dose HU (0.3 mM), the DNA damage checkpoint is not acutely induced, but replication forks are slowed, ribonucleotide reductase is upregulated and dormant replication origins are activated, enabling cell survival (11,44). Under these conditions, the spPCR signal of the ectopic $(CTG/CAG)_{102}$ repeats again was clearly reduced (Figure 1E).

In humans, CTG/CAG tracts above a threshold of 40–50 repeats are prone to expansion, whereas tracts with fewer repeats than the threshold are not associated with expansion (45). To determine whether microsatellite signal loss was dependent on repeat tract length, we constructed HeLa cells containing $(CTG/CAG)_{12}$ in place of $(CTG/CAG)_{102}$ at the same ectopic site. As shown in Fig-

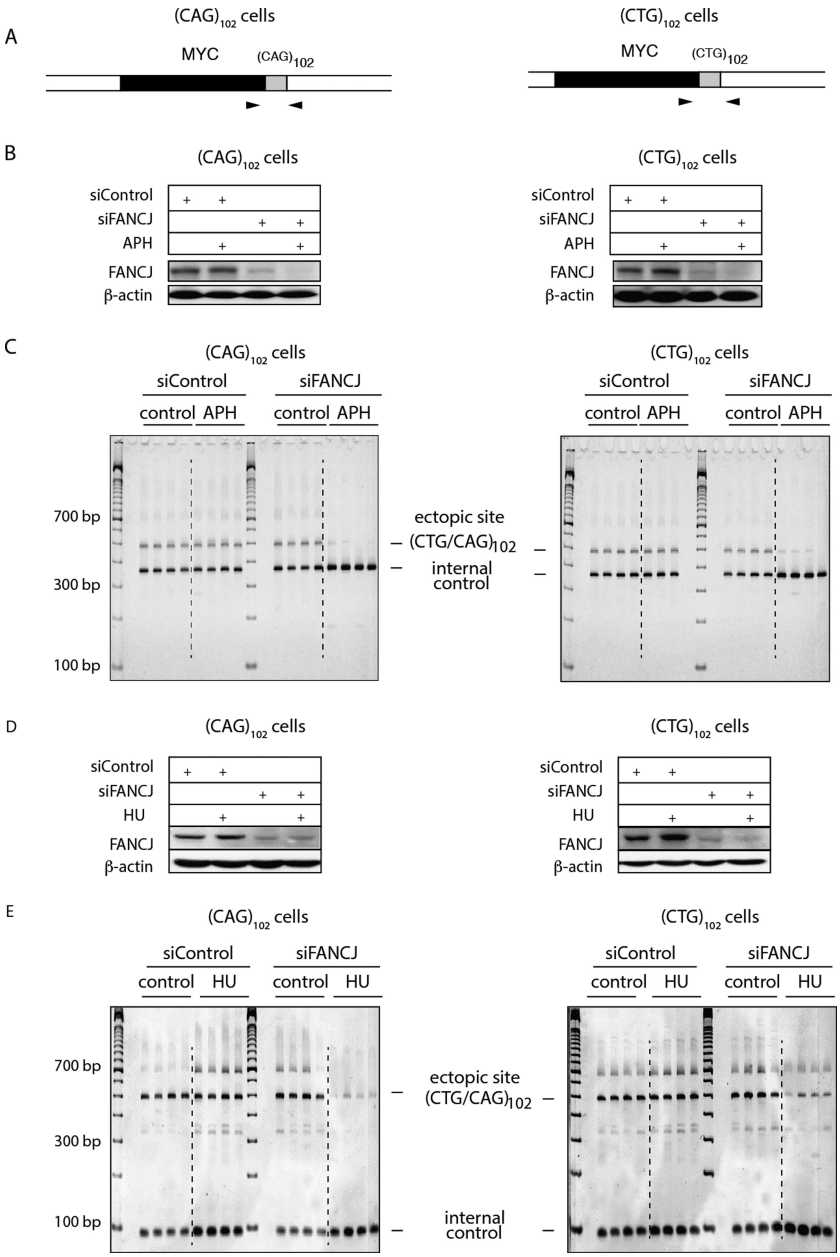

Figure 1. FANCJ knockdown leads to loss of ectopic CTG/CAG microsatellite signals in $(CTG/CAG)_{102}$ cells under replication stress. (**A**) Maps of the ectopic loci. (**B** and **D**) Western blots. Whole cell extracts were isolated after treatment of cells with siControl or siFANCJ for a total of five transfections, and either 0.2 uM aphidicolin, 0.2 mM hydroxyurea, or parallel untreated cultures and immunoblotted for FANCJ. (**C** and **E**) Duplex small pool PCR with primers spanning the ectopic $(CAG/CTG)_{102}$ repeats and primers for a non-repeat internal PCR control site.

ure 2, the $(CTG/CAG)_{12}$ microsatellite also displayed PCR signal loss when treated with FANCJ siRNA and APH, suggesting that (CTG/CAG) signal loss is not limited to expanded repeat tracts.

End-point spPCR can overestimate the amount of a low abundance template relative to a more abundant internal control whose amplification plateaus earlier in the PCR. To test the sensitivity of the spPCR assay, we titrated DNA from CTG_{102} cells containing one ectopic site and four internal control sites per genome (46,47) with control HeLa DNA (no ectopic copies). A four-fold dilution of the ectopic template by control DNA (from 1:4 copies to 1:16 copies) could readily be detected by end-point spPCR (Supplementary Figure S1H). We conclude that the decrease in spPCR signal from PCR primer binding sites closely adjacent to the ectopic CTG/CAG repeats occurs in a majority (>75%) of cells depleted of FANCJ and subjected to replication stress, although this does not preclude smaller changes in control cells below the limits of spPCR detection. Nevertheless, FANCJ depletion sensitizes cells to replication stress and results in loss of the ectopic $(CTG/CAG)_{102}$ and $(CTG/CAG)_{12}$ microsatellite signals from both leading and lagging strand templates irrespective of replication polarity.

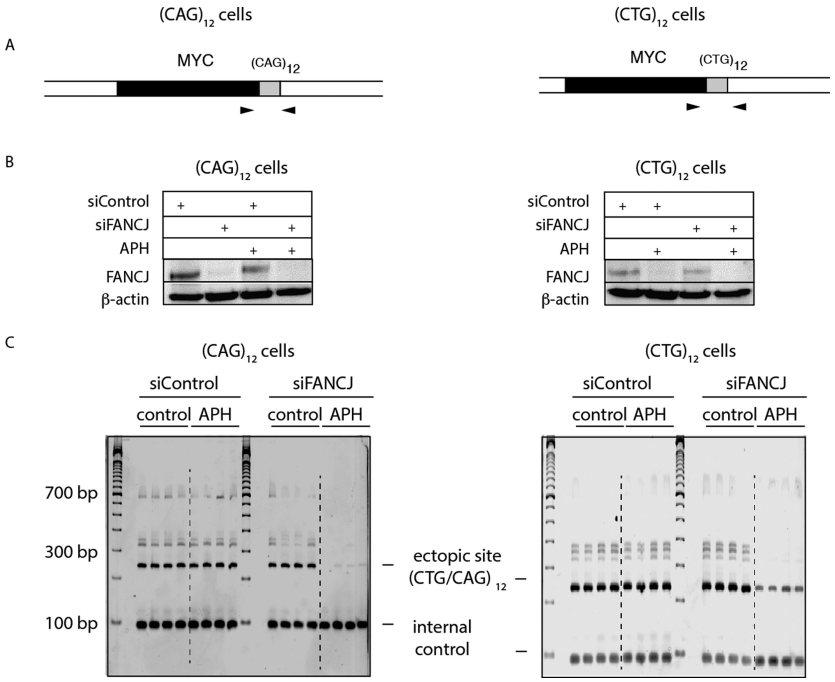

Figure 2. FANCJ knockdown leads to loss of ectopic CTG/CAG microsatellite signals in $(CTG/CAG)_{12}$ cells under replication stress (APH). (**A**) Maps of the ectopic loci. (**B**) Western blots. Whole cell extracts were isolated after treatment of cells with siControl or siFANCJ for a total of five transfections and 0.2 μM aphidicolin, or parallel untreated cultures and immunoblotted for FANCJ. (**C**) Duplex spPCR with primers spanning the ectopic $(CAG/CTG)_{12}$ repeats and primers for a non-repeat internal PCR control site.

FANCJ is required to maintain endogenous microsatellites against replication stress

The loss of the ectopic $(CTG/CAG)_{12}$ signal was surprising, and suggested that unexpanded, endogenous microsatellites might show a similar phenotype. To determine whether the instability of the ectopic CTG/CAG microsatellite repeats was unique, we analyzed repetitive sequences at several other loci [DMPK (CTG repeat, chr. 19.13.32), ATXN10 (ATTCT repeat, chr. 22q13.31), PKD1 (asymmetric pu/py mirror repeat, chr. 16p13.3), TUBA1B (G4 consensus, chr. 12q13.12), ERDA1 (CTG repeat, chr. 17q21.3), ATXN1 (CTG repeat, chr. 6p22.3), HBB (G4 consensus, chr. 11p15.4), TCF4 (CTG repeat, chr. 18q21.2), BRIP1 (poly T repeat, chr. 17q23.2), MYC (G4 consensus, 8q24.21), TP53 (poly T, 17p.13.1), TP63 (poly T, 3q28) in CAG_{102} cells (Supplementary Table S1)]. As observed for the ectopic site constructs, knockdown of FANCJ in the presence of APH (Figure 3) or HU (Supplementary Figure S2) also contributed to the much reduced spPCR signals of each of these endogenous repeats. It is not unexpected that there would be variability between the loss of signal at diverse sites if the presumed non-canonical structures are of different *in vivo* stability; nevertheless since endogenous CTG/CAG repeat signals at the DMPK, ERDA1, ATXN1 and TCF4 loci were lost following replication stress in FANCJ depleted cells, we conclude that gross microsatellite signal loss under these conditions is not limited to pathologically expanded CTG/CAG repeat tracts, consistent with the loss of the $(CTG/CAG)_{12}$ ectopic site signals (Figure 2).

In order to mitigate off-target effects, we used a pool of four siRNAs to knock down FANCJ. Significantly, three of the four FANCJ siRNAs individually led to the loss of microsatellite signals in cells treated with APH (not shown). As demonstrated in Supplementary Figure S2, siRNA knockdown of FANCJ with the pooled siRNAs also sensitized microsatellites to HU treatment. To confirm the observation that microsatellite signal instability was not an off-target effect of FANCJ siRNA transfection, FANCA null and FANCJ null patient cell lines from different FA patient cells were treated with APH. Both of these FA cell types are MMC sensitive, however, only the FANCJ null cells harboring a biallelic frameshift mutation were hypersensitive to APH treatment compared to wild-type FANCJ-proficient cells and to FANCA mutant cells (Supplementary Figure S1E). Similarly, CAG_{102} cells treated with FANCJ siRNA were also hypersensitive to APH (Supplementary Figure S1F).

As shown in Figure 4, APH treatment of FANCJ null cells resulted in the dramatic loss of spPCR signal from all of the tested microsatellite repeats relative to the internal controls. We have also observed loss of spPCR signal at the Friedrich's ataxia locus (FRN, chr. 9q21.11) GAA repeat and the myotonic dystrophy type 2 locus (ZFN9, chr. 3q21.3) CCTG repeat in these cells (not shown). FANCJ null cells treated with HU also displayed microsatellite signal instability (Supplementary Figure S4), reinforcing our findings that replication stress specifically causes microsatellite signal loss in FANCJ deficient or depleted cells.

It has been reported that the higher endogenous levels of RAD51 and RAD51 foci in $TP53^{-/-}$ cells (null for the p53 protein) than in $TP53^{+/+}$ cells can enhance HR follow-

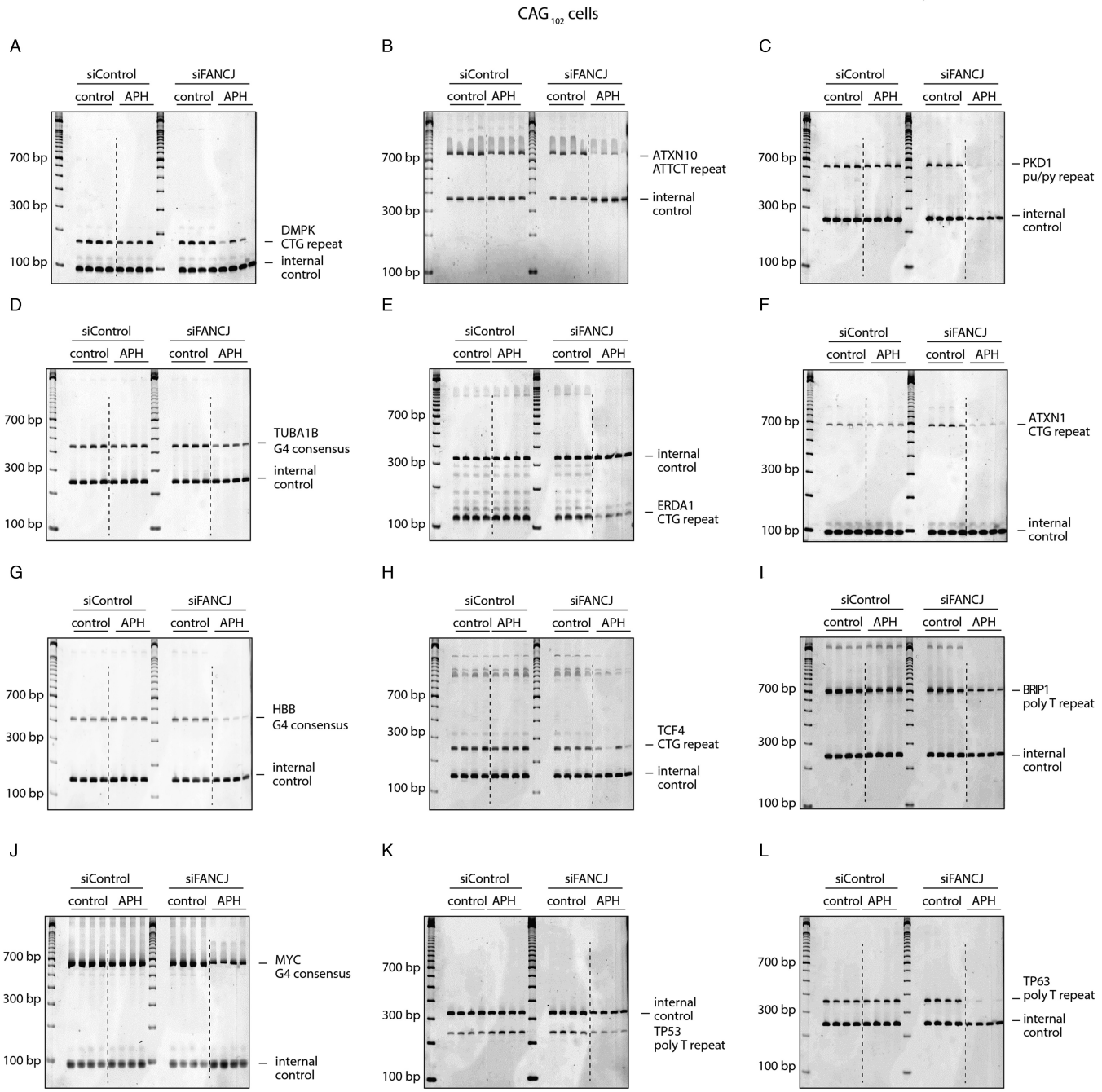

Figure 3. FANCJ knockdown leads to the loss of microsatellite signals at multiple endogenous sites under replication stress (APH). (**A**–**L**) Duplex spPCR across the endogenous DMPK (CTG repeat), ATXN10 (SCA10, ATTCT repeat), PKD1 (pu/py mirror repeat), TUBA1B (α-1B tubulin, G quadruplex), ERDA1 (CTG repeat), ATXN1 (SCA1, CTG repeat), HBB (β-globin, G quadruplex), TCF4 (CTG repeat), BRIP1 (FANCJ, poly T repeat), MYC (c-Myc, G quadruplex), TP53 (poly T repeat), TP63 (poly T repeat) loci in DNA from siControl or siFANCJ treated CAG_{102} cells (WB, see Figure 1B), with or without aphidicolin (0.2 μM) treatment.

ing replication stress (48,49). To determine whether p53 was downregulated in the FANCJ null cells, we compared p53 levels to those in wild-type fibroblasts and HeLa cells. As shown in Supplementary Figure S3M the results confirmed that p53 is downregulated in HeLa cells but expressed at similar levels in FANCJ-mutated cells and wild-type human fibroblasts. These results suggest that microsatellite signal loss during replication stress in FANCJ-mutated/depleted cells is independent of wild-type p53 levels.

Next, we tested whether the microsatellite signal instability seen during replication stress in FANCJ null cells was alleviated by re-introduction of wild-type FANCJ cDNA, which has been shown to enable replication fork restart after APH treatment (17). As shown in Figure 5, the microsatellite signal loss observed in FANCJ null cells under replica-

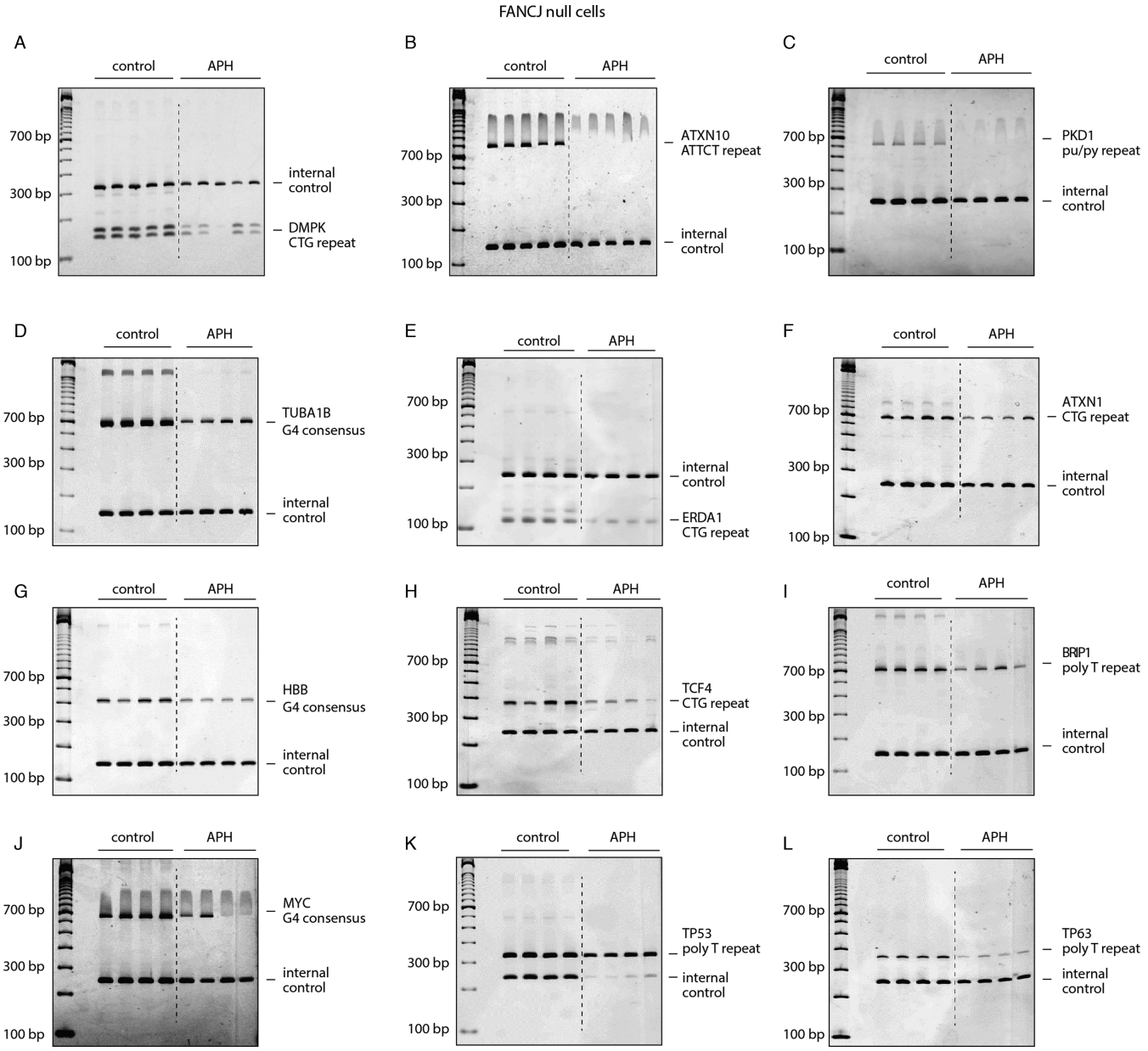

Figure 4. FANCJ$^{-/-}$ patient cells treated with aphidicolin are prone to microsatellite signal loss at multiple endogenous sites. (**A**–**L**) Duplex spPCR across endogenous repeated sequences in DNA from FANCJ$^{-/-}$ patient fibroblasts with or without aphidicolin (0.5 μM) treatment.

tion stress was completely reversed by expression of wild-type FANCJ protein (Supplementary Figure S4). This protection of microsatellites by WT FANCJ was also evident in the presence of HU as an alternative stressor (Supplementary Figure S4). In parallel as controls, normal human fibroblasts were also subjected to APH or HU treatment (Supplementary Figure S5). Neither of these conditions enhanced microsatellite signal instability in FANCJ wild-type expressing cells. We conclude that FANCJ depletion sensitizes the tested microsatellites to signal loss during treatment with APH or HU.

FANCJ can function independently of the FA pathway

In contrast to the effects of knocking down FANCJ, siRNA knockdown of the FANCM or FANCD2 proteins did not result in reproducible loss of CTG/CAG microsatellite signals in the presence of APH (Supplementary Figure S6). The loss of the ectopic CTG/CAG spPCR signals versus the internal control signals when FANCJ is knocked down suggests that depletion of FANCJ sensitizes cells to APH or HU induced replication stress independent of the remaining FA pathway.

Activation of the FANCD2 and FANCI proteins by mono-ubiquitination is a key step in the FA pathway for ICL repair, although these proteins may act independently during replication fork stalling (50). However, treatment of

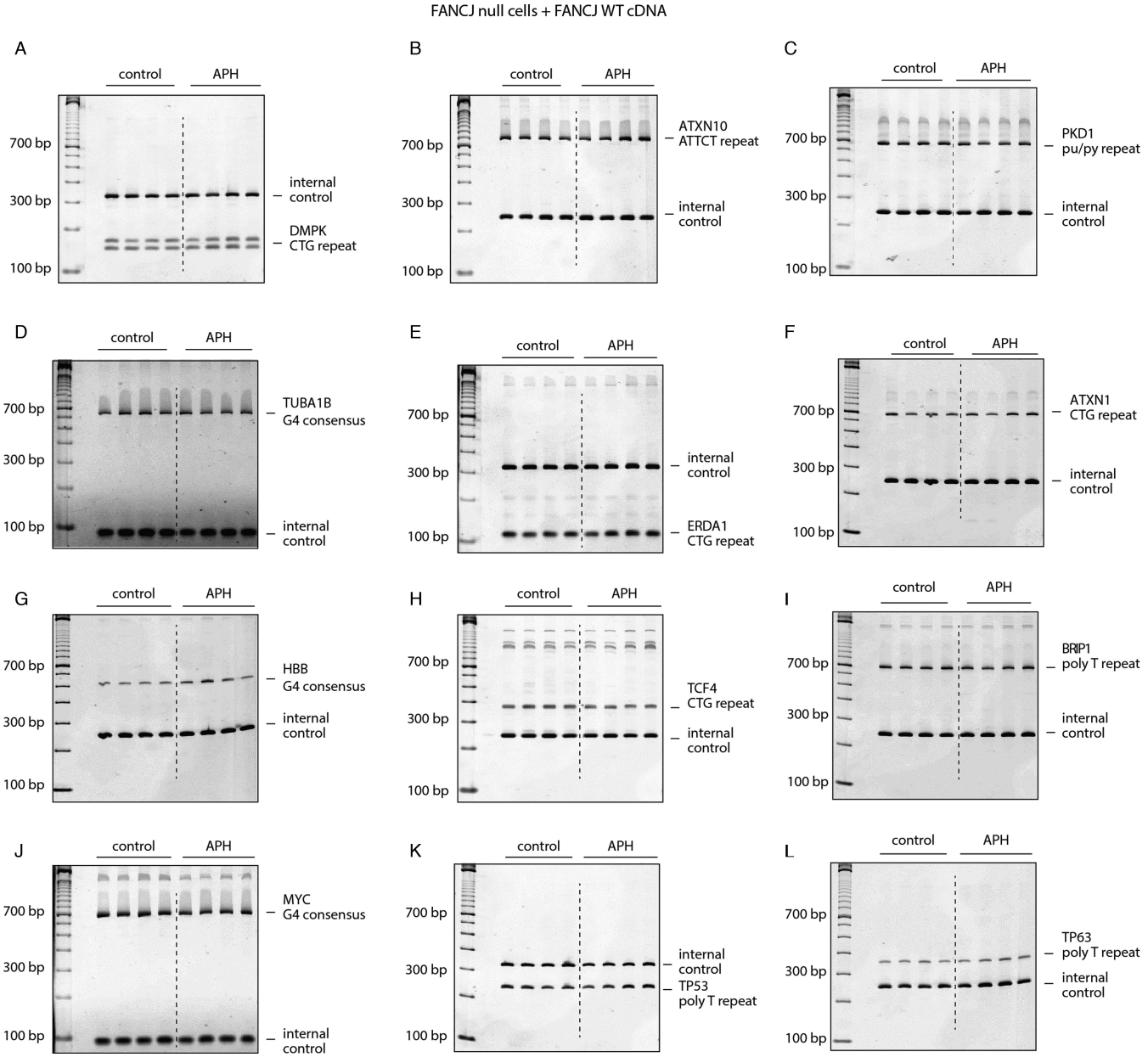

Figure 5. WT FANCJ cDNA expression reverses microsatellite signal instability of FANCJ$^{-/-}$ cells under replication stress (APH). (**A**–**L**) Duplex spPCR across endogenous repeats in DNA from FANCJ$^{-/-}$ patient fibroblasts expressing WT FANCJ cDNA and treated with or without 0.5 μM aphidicolin.

patient fibroblasts defective in an essential component of the core ubiquitin ligase, FANCA, with APH did not result in reproducible signal loss of the microsatellites tested (Supplementary Figure S7), nor did FANCD2 mutant cells display microsatellite signal loss after APH treatment (Figure 6). We also did not detect microsatellite signal instability after APH treatment of patient cells with biallelic defects in FANCI (Supplementary Figure S8), FANCD1 (Supplementary Figure S9), FANCP, a scaffold for binding the SLX1, MUS81 and XPF nucleases (Supplementary Figure S10), FANCC (Supplementary Figure S11), FANCQ (Supplementary Figure S12), FANCL, WRN or BLM, or in the presence of HU (not shown). Within the limits of spPCR detection, these results indicate that the FANCJ helicase has a unique role in the maintenance of diverse microsatellite DNAs independent of the activity of the other proteins in the FA pathway.

The most commonly occurring FA pathway mutation in FANCJ patient cells causes a truncation (FANCJ^{R798X}) that eliminates *in vitro* FANCJ helicase activity and removes the C-terminal binding sites for TOPBP1, BLM, MRE11 and BRCA1 repair proteins (21–23). Surprisingly however, ICL-sensitive FA cells containing the FANCJ^{R798X} truncation mutation were protected against microsatellite signal loss (Figure 7), suggesting that the C-terminal protein binding sites, and possibly the helicase activity, of FANCJ are not required to maintain these microsatellite repeats during replication stress.

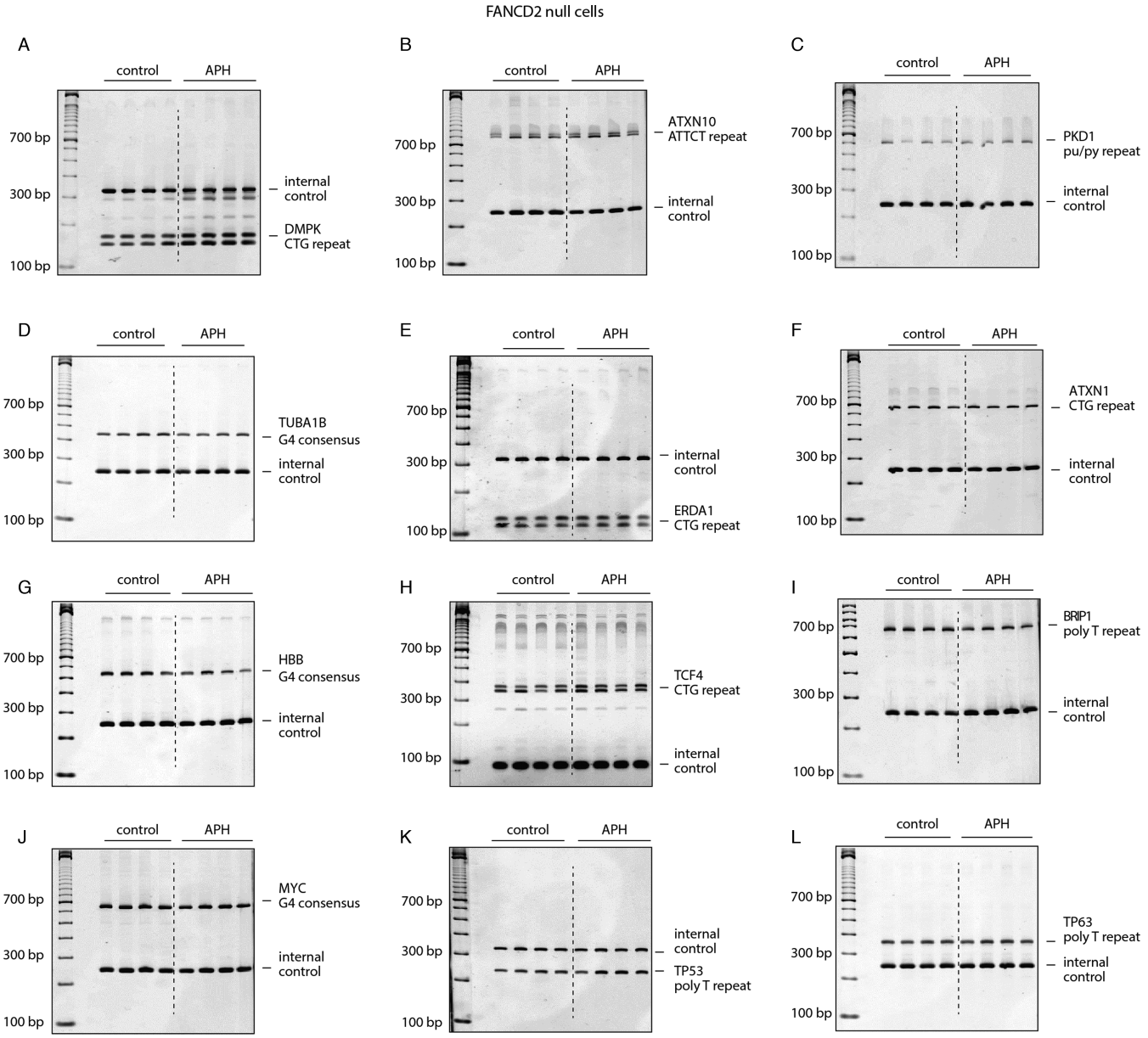

Figure 6. FANCD2 null cells do not show microsatellite signal loss under replication stress. (**A**–**L**) Duplex spPCR across endogenous repeated sequences in DNA from FANCD2 mutant patient fibroblasts with or without aphidicolin (0.5 μM) treatment.

Replication stress causes chromosome recombinations in FANCJ depleted cells

FANCJ null cells are prone to structural chromosome aberrations (1,2,23), a phenotype that is dramatically enhanced by exposure to DNA interstrand crosslinking agents (51,52). Based on the loss of spPCR signals at microsatellite repeats in FANCJ depleted cells exposed to replication stress, we hypothesized that DSBs in FANCJ mutant cells specifically occur in the vicinity of microsatellites and lead to chromosome recombination. We took an inverse PCR approach to test this hypothesis (Figure 8). Briefly, DNA was isolated from CTG_{102} HeLa cells treated with FANCJ siRNA and APH. The DNA was digested with the frequent cutting restriction enzyme MSE1 to cleave at the ectopic site and in the flanking DNA resulting from the presumed translocations. The DNA was self-ligated at low concentration and PCR primers oriented outward from the ectopic site (inverse PCR, iPCR) were used to amplify the circularized DNA, which was analyzed by DNA sequencing.

This strategy does not distinguish between the great majority of iPCR product reads that were not rearranged from those that were repaired by sister chromatid HR. However, next generation sequencing revealed that ∼28 000 reads out of a total ca. 26 million reads contained ectopic site junctions with other chromosomes (Figure 8). These junctions occurred non-randomly over the genome. As expected, the most frequently detected recombination event was the result of non-allelic HR (NAHR) between c-myc sequences at the ectopic site (chromosome 18) and the endogenous

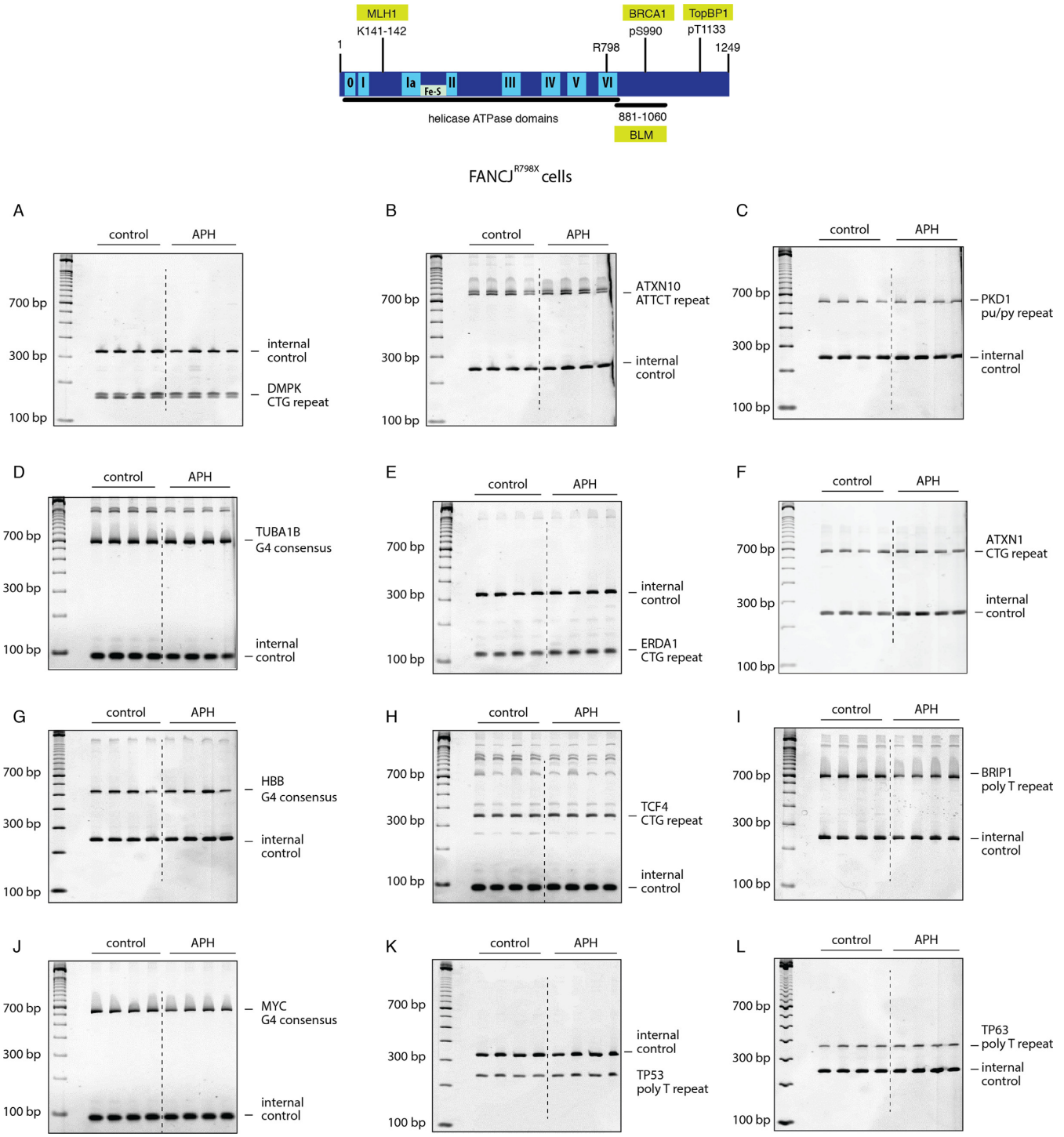

Figure 7. FANCJ^{R798X} cells treated with aphidicolin are not prone to microsatellite signal loss. Upper panel, schematic diagram of FANCJ. Lower panels **A**–**L**, duplex spPCR across endogenous repeated sequences in DNA from FANCJ$^{-/-}$ patient fibroblasts with or without aphidicolin (0.5 μM) treatment.

c-myc locus at chromosome 8q24.21. We also observed recombination of the ectopic site $(CTG)_{102}$ trinucleotide repeat with the $(CTG)_{20}$ trinucleotide repeat in the 3′ UTR of the dystrophia myotonica protein kinase gene (chromosome 19q13.32). Hence the ectopic site trinucleotide repeat may also undergo NAHR under these conditions. Other sites that show no more than microhomology to the ectopic site were also involved in these non-random translocations (see 'Discussion' section). These results support the view that depletion of FANCJ potentiates the appearance of double strand breaks under replication stress, that result in chromosome recombination at microsatellite sequences.

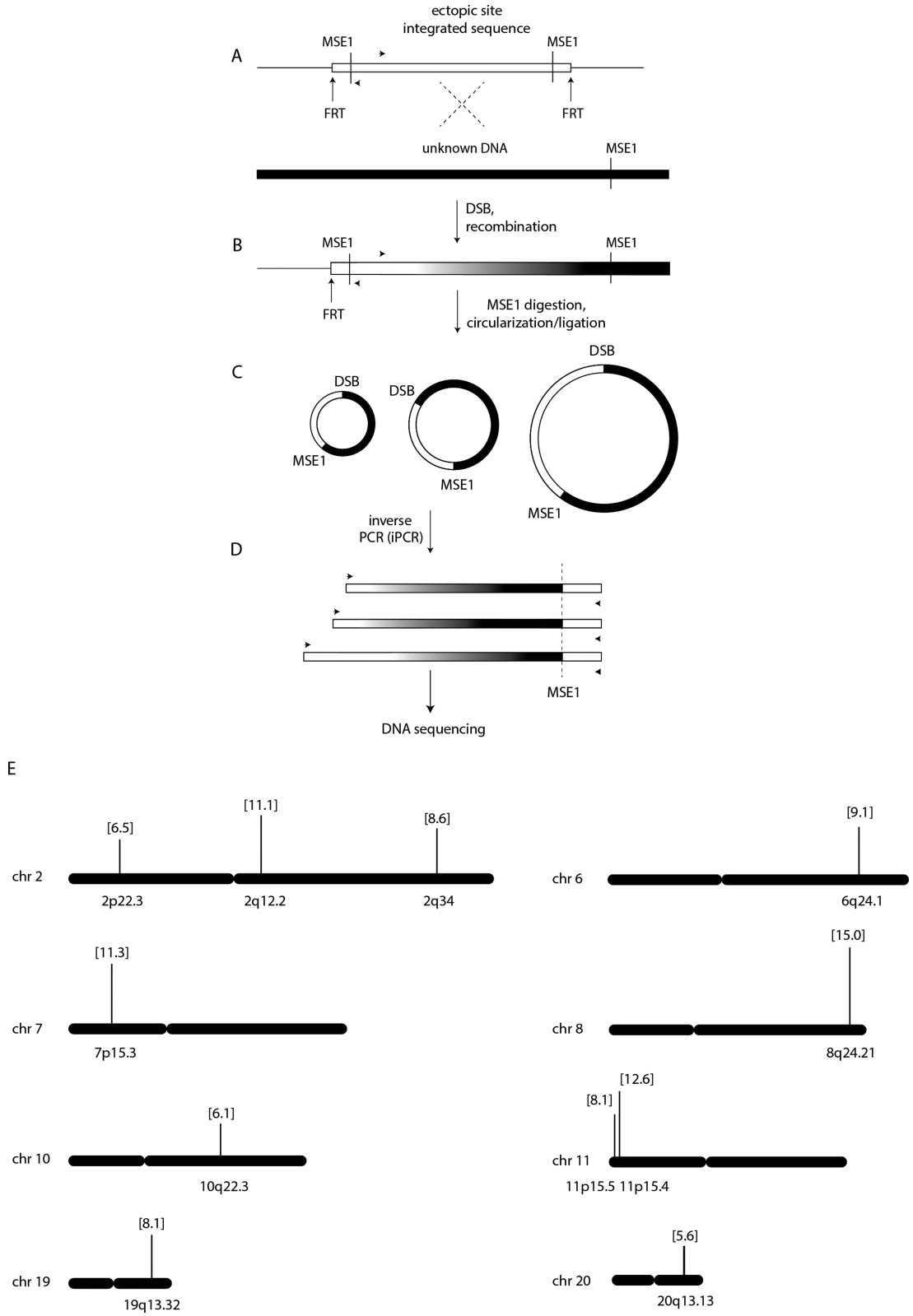

Figure 8. Inverse PCR mapping of ectopic site CTG/CAG microsatellite recombination. (**A** and **B**) Model of ectopic site double strand break and recombination. Gradient fill indicates multiple potential recombination sites; divergent arrowheads show the locations of the iPCR primers. (**C**) Circular DNAs after MSE1 digestion and intramolecular ligation. (**D**) iPCR products. (**E**) Cytogenetic locations of DNA sequencing reads (bars and bracketed values = $\log_2$ read copy number) containing chromosomal translocation junctions with ectopic site sequences.

DISCUSSION

Instability of microsatellite tracts by expansion of repeat number contributes to many human diseases by interference with transcription, RNA processing and protein translation (53). In cell culture, expansion or contraction of CTG/CAG TNRs can be induced by recurrent cycles of replication stress and recovery (33,34,43,54,55). Here we show that CTG/CAG TNRs are also sites of apparent chromosome fragility in FANCJ depleted cells during continuous, prolonged replication stress. Thus, knockdown of FANCJ results in loss of ectopic site $(CTG)_{102}$ and $(CAG)_{102}$ spPCR signals when replication is slowed by low dose aphidicolin or HU. Surprisingly, many different endogenous microsatellite sequences with the common tendency to adopt non-canonical DNA structures also show spPCR signal loss when FANCJ is knocked down by siRNA under replication stress. Thus, this phenotype is not limited to expanded trinucleotide repeats but occurs at diverse microsatellites widespread across the genome.

We considered several alternatives to DNA DSBs as explanations for spPCR microsatellite signal loss. One is a jackpot increase in repeat length beyond the limit of efficient PCR. However, we have demonstrated PCR of CTG/CAG tracts of >1000 repeats (43) and spPCR signals are lost from microsatellites that are not known to expand (e.g. MYC, HBB, TUBA1B G4 consensus sequences; the PKD1 pu/py tract; DMPK $(CTG/CAG)_{12\text{-}20}$; ectopic site $(CTG/CAG)_{12}$). Another possibility is that PCR primer binding sites are deleted. Not only would this implicate DSBs, but primer binding sites 1–3 kb distal to the ectopic site are present in FANCJ depleted cells after APH, but cannot be used to amplify across the ectopic site (Supplementary Figure S1). Finally, it is conceivable that microsatellites under replication stress form a structure *in vivo* that cannot be amplified by in vitro PCR. We dismiss this explanation since the hypothetical structure would have to be resistant to deproteinization, DNA purification and repeated thermal denaturation. Moreover, when more than 50% of microsatellite signal is lost, both template strands would have to become PCR resistant and this effect would have to occur at multiple different microsatellites. Taken with evidence of interchromosomal recombination between the ectopic and endogenous microsatellites, we propose that repeated DNAs undergo DNA DSBs as a result of replication stress in FANCJ depleted cells.

The same microsatellites were hypersensitive in FANCJ depleted HeLa cells and in FANCJ$^{-/-}$ fibroblasts, but not in cells deficient in FA proteins upstream (FANCA, -C, -D2, -I, -L or downstream (FANCD1, -Q, -P) in the FA pathway. This observation opens the possibility of using microsatellite instability as a diagnostic test to distinguish the FANCJ complementation group from other forms of FA. Considered with the observation that FANCJ$^{-/-}$ patient cells display an increased level of chromosome aberrations under naturally occurring replication stress (56,57), our results suggest that microsatellites might be inherently prone to breakage during replication and that FANCJ has evolved to prevent microsatellite DSBs during endogenous replication stress.

The absence of microsatellite instability after expression of WT FANCJ in FANCJ$^{-/-}$ cells argues that FANCJ deficiency is responsible for the cellular DNA instability phenotype. Moreover, microsatellite loss in these cells does not result from a collateral decrease in WRN or BLM proteins since WRN and BLM null patient cells do not display the same microsatellite sensitivity phenotype. Moreover, loss-of-function germ-line mutations of other FA proteins did not elicit the same phenotype, thus FANCJ specifically functions outside the intact FA pathway to stabilize microsatellites. The FANCJ helicase is active *in vitro* in unwinding non-canonical DNA structures that block DNA synthesis, and in HR and the removal of DNA ICLs. However, it remains to be determined whether FANCJ stabilizes microsatellites *in cellulo* by resolving DNA template stall sites (25), by promoting fork regression and template switching (58,59), or by enabling HR (21).

FANCJ deficiency leads to general microsatellite instability under replication stress. We emphasize that each of the observed translocation sites has previously been identified cytogenetically as a site of chromosome instability associated with developmental disorders and cancers (chr. 2p22.3, small cell lung carcinoma (60); chr. 2q12.2, small cell lung carcinoma (60); chr. 2q34, breast cancer (61,62); chr. 6q24.1, breast cancer, neuroblastoma, melanoma (63); chr. 7p15.3, ovarian cancer (64,65); chr. 8q24.21, leukemia/lymphoma (66,67); chr. 10q22.3, non-small cell lung cancer, prostate cancer (68,69); chr. 11p.15.4-5, Beckwith-Wiedemann cancer predisposition syndrome, T lymphoid leukemia (70–72); chr. 20q13.13, hepatocellular carcinoma (73)). We note as well that at the nucleotide level most of the NGS reads with recombination junctions contain structure-prone sequences predicted to be unstable under replication stress (G_{300}, 2p22.3; G4, 2q12.2, 2q34, 7p15.3, 8q24.21, 11p15.4, 20q13.13; T_{20}, 6q24.1; CTG_{20}, 19p13.32). Thus, we speculate that concurrent microsatellite DSBs generate a pool of DNA ends that are substrates for NHEJ/MMEJ and chromosome recombination, or form structures that are not efficiently processed by classical DSB repair mechanisms. The high frequency of microsatellite DSBs in FANCJ depleted cells under replication stress leads us to propose that the replication of non-canonical DNA structures may be a source of chromosome abnormalities in cases of inherited or stochastic FANCJ insufficiency.

NOTE ADDED IN PROOF

Results consistent with those presented here have recently been published by Matsuzaki *et al*. (74).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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