Deficiency of the Fanconi anemia E2 ubiquitin conjugase UBE2T only partially abrogates Alu-mediated recombination in a new model of homology dependent recombination

Todd W. Lewis1, Joanna R. Barthelemy1, Elizabeth L. Virts2, Felicia M. Kennedy2, Rujuta Y. Gadgil1, Constanze Wiek3, Rene M. Linka3, Feng Zhang4, Paul R. Andreassen4,5, Helmut Hanenberg3,6,† and Michael Leffak1,*†

1Department of Biochemistry and Molecular Biology, Boonshoft School of Medicine, Wright State University, Dayton, OH, USA, 2Department of Pediatrics and Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN 46202, USA, 3Department of Otorhinolaryngology and Head/Neck Surgery, Heinrich Heine University, 40225 Duesseldorf, Germany, 4Division of Experimental Hematology & Cancer Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH, USA, 5Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA and 6Department of Pediatrics III, University Children’s Hospital Essen, University of Duisburg-Essen, 45122 Essen, Germany

Received November 11, 2017; Revised January 04, 2019; Editorial Decision January 07, 2019; Accepted January 30, 2019

ABSTRACT

The primary function of the UBE2T ubiquitin conjugase is in the monoubiquitination of the FANCI-FANCD2 heterodimer, a central step in the Fanconi anemia (FA) pathway. Genetic inactivation of UBE2T is responsible for the phenotypes of FANCT patients; however, a FANCT patient carrying a maternal duplication and a paternal deletion in the UBE2T loci displayed normal peripheral blood counts and UBE2T protein levels in B-lymphoblast cell lines. To test whether reversion by recombination between UBE2T AluYa5 elements could have occurred in the patient’s hematopoietic stem cells despite the defects in homologous recombination (HR) in FA cells, we constructed HeLa cell lines containing the UBE2T AluYa5 elements and neighboring intervening sequences flanked by fluorescent reporter genes. Introduction of a DNA double strand break in the model UBE2T locus in vivo promoted single strand annealing (SSA) between proximal Alu elements and deletion of the intervening color marker gene, recapitulating the reversion of the UBE2T duplication in the FA patient. To test whether UBE2T null cells retain HR activity, the UBE2T genes were knocked out in HeLa cells and U2OS cells. CRISPR/Cas9-mediated genetic knock-out of UBE2T only partially reduced HR, demonstrating that UBE2T-independent pathways can compensate for the recombination defect in UBE2T/FANCT null cells.

INTRODUCTION

Alu elements are the most abundant short interspersed elements (SINEs) in the human genome, numbering over one million copies. These repetitive sequences are hotspots for genetic intrachromosomal or interchromosomal recombination (1). The proximity of abundant Alu elements in the genome clearly favors deletions by RAD51-independent intrachromosomal single strand annealing (SSA) (2). Alu-mediated recombination (AMR) events contribute to multiple forms of cancer and other genetic disorders (3–8), and are estimated to be responsible for 0.3% of human genetic diseases (4,9). These repeated elements also drive genomic evolution; it has been estimated that more than five hundred Alu-mediated deletion events have occurred since divergence of the human and chimpanzee genomes (9). Here, we modeled an unusual somatic reversion event in a Fanconi anemia (FA) patient who had inherited a partial genomic duplication in the FANCT/UBE2T gene from his mother. In the current model system, an in vivo double strand break leads to homology-dependent recombination between two UBE2T Alu elements, mimicking a contraction of the maternal duplication to restore the WT allele. FA is a rare recessive or dominant DNA repair disorder characterized by genome instability, developmen-

1To whom correspondence should be addressed. Tel: +1 937 422 0801; Email: michael.leffak@wright.edu
2The authors wish it to be known that, in their opinion, the last two authors should be regarded as Joint Corresponding Authors.

© The Author(s) 2019. Published by Oxford University Press on behalf of Nucleic Acids Research. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
tal abnormalities, bone marrow failure and cancer predisposition (10–12). Loss-of-function mutations in one X-chromosomal (FANCB) and at least twenty autosomal recessive genes (FANCA to RFWD3/FANCW) as well as missense mutations in one dominant negative FA gene (RAD51A/FANCR) result in the typical defects associated with FA (13–15). At the cellular level in FA deficient cells, genome instability in combination with erroneous repair of DNA interstrand crosslinks (ICLs) and DNA double strand breaks often results in complex genome rearrangements (CGR), translocations and gene amplification (16–18). Among the known activities of FA proteins are replisome stabilization during replication stress (17,19), the removal of DNA ICLs caused by endogenous aldehydes (20), the resolution of R-loops (21), stimulation of the alternative end joining (Alt-EJ)/microhomology-mediated end joining (MMEJ) of DNA double strand breaks (22), regulation of the spindle assembly checkpoint (23,24), and autophagic clearance of damaged mitochondria or viruses (25).

The diagnosis of FA is based on the combination of typical clinical symptoms and the characteristic hypersensitivity of cells from affected patients to the ICL reagents diepoxybutane (DEB), mitomycin C (MMC), melphalan or cisplatin, which are often used to dissect the functions of individual FA proteins (18,26). A key step in activating ICL repair is the monoubiquitination of FANCI and FANCD2 in the FANCI-FANCD2 (ID2) protein complex by the thirteen subunit FA core complex containing FANCL as the E3 ubiquitin ligase (reviewed in (26,27)). The FANCT/UBE2T gene product is not part of this protein complex but encodes the major E2 ubiquitin conjugating enzyme used by the FANCL E3 ligase to modify and activate the DNA-bound ID2 dimer (28–31). Monoubiquitination of FANCI and FANCD2 is necessary for their co-localization into nuclear foci. Additional roles for FANCI and FANCD2 in the stabilization of replication forks and HR have also been reported (17,30,32–35).

Machida et al. (36) and Alpi et al. (37) have shown that UBE2T is the E2 conjugating ligase in the FA pathway and that genetic deficiency in UBE2T−/− DT40 cells leads to the classical cellular phenotypes of FA, including hypersensitivity to low doses of DNA ICL agents and high frequencies of chromosomal abnormalities. Subsequently, three groups including ours independently described three FA patients with germ-line defects in the UBE2T gene, now also designated FANCT (18,38–40). The 16-year-old FA patient (100166/1) of Italian ancestry described by us (40) was born with bilateral malformations of both thumbs and radii, microcephaly, café-au-lait spots and left kidney abnormality. He was confirmed as being affected by FA due to high levels of DEB-induced chromosomal breakage in metaphases of peripheral blood lymphocytes at birth (40). We identified the patient’s primary fibroblast cells as being defective in UBE2T by overexpression of the wildtype UBE2T cDNA as a candidate FA gene (RefSeq: NM_014176.3) which entirely corrected G2/M phase arrest and also other cellular phenotypes induced by MMC. Importantly, no mutation in the UBE2T locus could be detected in the patient’s germ-line DNA by Sanger sequencing or next-generation sequencing of UBE2T, as he had inherited genomic rearrangements at the two identical 311-bp AluYa5 elements present in the same orientation in introns 1 and 6 of the human UBE2T gene.

Notably, three Alu-mediated recombination events were evident at the UBE2T locus In the FANCT−/− 100166/1 proband (40). From his heterozygous father, the patient had inherited a large genomic deletion of exons 2–6, resulting in an allele without any protein-coding transcript. From his healthy mother, the patient inherited a UBE2T allele in which a duplication of exons 2–6 had occurred, resulting in a UBE2T locus with three identical AluYa5 repeats. Importantly, this maternal allele was capable of expressing a transcript for a truncated UBE2T protein that contained the complete ubiquitin binding (UB) domain of UBE2T (40). When overexpressed, this shorter protein completely restored the defects in the FA pathway in UBE2T−/− cells (40). However, western blot analysis revealed that no mutant UBE2T protein was expressed from the duplicated maternal allele in either the patient’s or his mother’s cells, as the mRNA from this allele was subject to nonsense mediated RNA decay (40). The third recombination event in the UBE2T locus occurred somatically in utero in a hematopoietic stem cell, as the patient’s peripheral blood lymphocytes were already a mixture of normal and FA-deficient cells when analyzed by chromosomal breakage three days after birth (40). Here, it is safe to hypothesize that the normal UBE2T allele was generated by intrachromosomal SSA or unequal sister chromatid homologous recombination between the maternally duplicated Alu elements (Figure 1A), as no normal allele that could serve as a recombination donor is present in the patient’s cells. Sequencing of FANCT−/− 100166/1 proband genomic DNA PCR products corroborated that the reversion had occurred at the AluYa5 repeats within the UBE2T locus (40). Subsequently, this ‘corrected’ hematopoietic stem cell repopulated the entire hematopoietic system with normal progeny - a phenomenon known as somatic mosaicism in FA (41)—and the patient had normal peripheral blood counts for more than 15 years and never experienced bone marrow failure.

The two main branches of homology directed recombination (HDR) are RAD51-independent single strand annealing (SSA) (42) and RAD51-dependent homologous recombination (HR) (43). To develop a model to emulate Alu-mediated homology directed recombination events in the UBE2T locus and for other loci in the genome, we generated dual fluorescent reporter constructs using two independent expression cassettes for green (eGFP) and red (dTomato) fluorescent proteins with three identical AluYa5 repeats in the same orientation. An exogenous I-Sce1 site was included at either of two distinct locations in the reporter constructs to allow introduction of a single site-specific DNA double strand break (DSB). After stable integration of one copy of the dual fluorescent reporter construct into the genome of HeLa cells, we show that expression of the I-Sce1 protein in the cells promotes DNA breakage and homology-directed AMR that mimics the reversion which had happened in the patient’s hematopoietic cells.

Using the dual fluorescence system, we find that UBE2T has a limited role in HR, and demonstrate the role of HDR in Alu-mediated recombination in UBE2T using inhibitors or knockdown of HDR or nonhomologous end joining (NHEJ)-related proteins. Our results show that the dual flu-
Figure 1. Modeling of the expanded UBE2T locus in dual fluorescence (DF) cells. (A) Wild type UBE2T gene, and maternal and paternal genotypes. Exons are numbered 1–7; yellow boxes, AluYa5 sequences; unfilled boxes, noncoding exons; filled boxes, coding exons. (B) DF1 cells contain a single genomic integrant at the FLP recombinase target (FRT) site in HeLa/406 cells, containing three identical AluYa5 repeats (yellow) and portions of intervening sequences IVS 1 and IVS 6 flanking the Alu repeats (blue boxes). The first and second Alu repeats are separated by a dTomato fluorescent protein gene (red) driven by the hPGK promoter; the second and third Alu repeats are separated by an eGFP gene (green) driven by the SFFV U3 promoter. In the DF2 cell line, an I-SceI cleavage site separates the dTomato and eGFP marker genes. In the DF3 cell line, an I-SceI cleavage site is located upstream of the dTomato marker gene. Thin lines, vector sequences. hygR (hygromycin resistance), neoR (G418 resistance) and TK (HSV thymidine kinase, ganciclovir sensitivity) are selection markers for integrant construction (Methods).

MATERIALS AND METHODS

Dual Fluorescence (DF) plasmid constructs

The plasmids used in this work were constructed to avoid regions of homology with the resident vector at the ectopic integration site (44) or within the dual fluorescence vectors other than the Alu/IVS elements. Standard cloning methods were used to construct the vector integrated in DF3 cells from the following components: LacZ (nt 1–120, 6917–7374); AluYa5 (nt 135–445); UBE2T intron 1 (nt 446–626); I-SceI recognition (nt 632–662); hPGK promoter (nt 663–1204); dTomato (red fluorescent protein, nt 1222–1927); bGHpA (nt 1936–2152); AluYa5 (nt 2167–2478); UBE2T intron 6 (nt 2478–2658); SFFV U3 promoter (nt 2696–3036); eGFP (enhanced green fluorescent protein, nt 3072–3792); thymidine kinase; and FRT site (nt 4724–4772); neomycin phosphotransferase gene (nt 4781–5576); SV40 polyadenylation sequences (nt 5577–6917); chloramphenicol acetyltransferase gene (nt 7822–8482); pSC101 origin of replication and RepA binding site (nt 10298–9406). Plasmids used to construct the vector integrated in DF3 cells from the following components: LacZ (nt 1–120, 6917–7374); AluYa5 (nt 135–445); UBE2T intron 1 (nt 446–626); I-SceI recognition (nt 632–662); hPGK promoter (nt 663–1204); dTomato (red fluorescent protein, nt 1222–1927); bGHpA (nt 1936–2152); AluYa5 (nt 2167–2478); UBE2T intron 6 (nt 2478–2658); SFFV U3 promoter (nt 2696–3036); eGFP (enhanced green fluorescent protein, nt 3072–3792); thymidine kinase; and FRT site (nt 4724–4772); neomycin phosphotransferase gene (nt 4781–5576); SV40 polyadenylation sequences (nt 5577–6917); chloramphenicol acetyltransferase gene (nt 7822–8482); pSC101 origin of replication and RepA binding site (nt 10298–9406). Plasmids used

fluorescent HeLa cells are also a robust tool for the systematic study of Alu-mediated recombination events and their role in inducing human disease. Combined with knockdowns of specific genes of interest, the dual fluorescence system can quantitatively report on the contribution of specific proteins to HDR and NHEJ.
to construct other cell lines were derived from the same components. Further details are available from the authors. As shown in Supplementary Figure S1 and confirmed by the present results, there is insufficient homology between the red and green fluorescent protein genes to enable homologous recombination.

Cell culture

DF cell lines were constructed by FLP recombinase mediated integration into HeLa/406 cells and drug selection as described previously (45–50). All DF cell lines were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco) supplemented with 10% newborn calf serum (NCS) at 37°C, 5% CO2. The human osteosarcoma (U2OS, ATCC HTB-96) DR-GFP cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C, 5% CO2.

Expression of I-SceI endonuclease

Stable DF cell lines plated at 6 x 10^5 cells were trypsinized and transfected with 8 ug I-SceI plasmid and 10 μl of Lipofectamine 2000 (Invitrogen) per manufacturer’s protocol in a six-well plate (Falcon). Medium was replaced after 24 h to remove transfection complexes. The I-SceI plasmid produces an HA-tagged form of the I-SceI endonuclease. Peak expression of I-SceI endonuclease was at 24 h and was undetectable after 72 h by western blot (Supplementary Figure S2). Cells were grown for 8 days after I-SceI transfection and split accordingly until harvested for flow cytometry. The I-SceI plasmid was a gift from John Turchi (Indiana University School of Medicine).

siRNA treatment

DF2 cell lines were plated at 4 x 10^5 cells/well in a six-well tissue culture plate (Falcon). The cells were trypsinized and transfected with 100 nM siCtIP siRNA (Hs_SI03650318). At 24 h, cell cultures were split and centrifuged at 300 g for 3 min. Supernatant was removed and the cell pellet was resuspended in 300 μl FACS buffer (Hank’s Balanced Salt Solution, 25 mM HEPES, 1 mM EDTA, 1% BSA and 2% FBS) and filtered through a 35 μm cell strainer tube (Falcon). Cell sorting was performed at the Cincinnati Children’s Hospital Medical Center (CCHMC) Research Flow Cytometry Core (RFCC) on a BD FACS Aria II flow cytometer with two 96 well tissue culture plates (single cell per well) (Corning) for each of the four flow cytometry quadrants. Single cell clones from each well were transferred to 10 cm tissue culture dishes (Corning). Once the 10 cm dishes were ~80% confluent the cells were harvested for DNA analysis. DNA was isolated using an EZNA tissue isolation kit (Omega Bio-Tek) to serve as template in PCR amplifications.

Polymerase chain reaction

PCR was performed using Lac-Forward (5′-CTTCAA ATCCGACCCTAGA-3′) and TK-Reverse (5′-GTAAG GTCATCGGCTGGGTA-3′) primers. PrimeSTAR GXL polymerase (TaKaRa) was used per manufacturer’s instructions for 50 μl reactions using 120 ng template. Internal control primers (Figure 3) were: forward 5′-ACCTACACGTTCTAAC and reverse 5′-CCAGCAGAAC CGTCTCATAAT. Cycling conditions were as follows: denaturation, 98°C, 10 s; annealing, 57°C, 15 s; extension, 68°C, 1 min for 30 cycles. PCR products were purified with an EZNA Cycle Pure kit (Omega Bio-Tek) and 20 μl of each purified product was electrophoresed on a 1% ultrapure agarose gel (Invitrogen) to verify the sizes of the recombination products.

Flow cytometry

Flow cytometry was performed on the BD Accuri C6 flow cytometer. 5 x 10^5 adherent cells were trypsinized (Gibco) and centrifuged at 300 x g for 3 min. Supernatant was removed and the cell pellet was resuspended in 300 μl PBS. The cells were centrifuged for another 3 min at 300 x g. The supernatant was aspirated and the cell pellet was resuspended in 200 μl PBS. The number of events counted was set to 20 000 on medium flow. Color compensation was set at correcting FL2 by subtracting 6.3% of FL1. The color compensation and quadrant determination was determined empirically to minimize spectral overlap using marker DF6 and DF7 cell lines that produce only red fluorescent dTomato protein or green fluorescent eGFP protein respectively.

CRISPR-Cas9 knockdown

CRISPR guides that target UBE2T were selected using the design website http://crispr.mit.edu (51). Guide 2 (TTTGATACCTACGAGCTCGCAG) was chosen after
single cell cloning of transduced cells and western analysis. This guide RNA is complementary to intron 1, approximately 1.3 kb downstream of the *UBE2T* 5′ Alu element. Briefly, LentiCRISPR v2 containing the puromycin resistance-mediating pac gene was a kind gift from Feng Zhang (Addgene plasmid #52961). Cloning of the guide RNA into the LENTICRISPR v2 vector was performed as specified by the Zhang laboratory protocol (52). Infectious replication-deficient lentiviral particles in the VSV-G pseudotype were generated as previously described (40). DF3 and U2OS DR-GFP cell lines were transduced with a lentivirus construct expressing Cas9 and the *UBE2T* CRISPR sgRNA at an MOI of 1.3. The first Alu element is located on the plus strand with the translation start in exon 2, and two identical *UBE2T* wild-type alleles in humans comprises seven exons with the translation start in exon 2, and two identical AluYa5 elements in the same orientation (www.ensembl.org). The first Alu element is located ∼180 bp 3′ of the exon1/intron 1 border, and a second Alu element is ∼180 bp 3′ of the exon 6/intron 6 border (Figure 1A). The mutant maternal allele contains a duplication of exons 2–6 and the mutant paternal allele exhibits a deletion of exons 2–6. Both mutant alleles seem to have occurred by Alu-mediated recombination. We have shown previously that the maternal allele is present as a founder mutation at low frequencies in the Italian and the German populations. However, we did not detect the maternal duplication in almost 2000 individuals (40) suggesting that it is much more restricted to the maternal lineage. In the proband, spontaneous deletion of the maternally duplicated exons 2–6 by recombination between neighboring Alu elements has been postulated to account for the outgrowth of normal blood cells and the reversion of the hematopoietic phenotypes (40).

To simulate Alu-mediated recombination in the *UBE2T* locus, we generated clonal cell lines in which one copy of a model *UBE2T* locus was integrated into the single FLP recombination target (FRT) site in HeLa/406 cells (45–49,53) (Figure 1B). Previous work has shown that Alu-mediated recombination is responsible for homozygous deletions in the *STK11/LKB1* locus of HeLa cells to generate aberrant LKB1 fusion transcripts (54), for *MLL/KMT2A* duplications in normal and AML hematopoietic cells (55), and for nonallelic homologous recombination deletions in the FA, B, C and D2 genes (5,56) in FA patient cells. To model the maternal *UBE2T* allele containing the duplication, three AluYa5 repeats and ∼180 bp of *UBE2T* flanking intron 1 (IVS1) or intron 6 (IVS6) sequences were separated by complete reporter gene cassettes encoding the red fluorescent protein (RFP) and the human phosphoglycerate kinase (PGK) promoter with a 3′ bGH polyA site, and the eGFP protein driven by a modified viral SFFV U3 promoter region followed by the polyA sequences of a SIN lentiviral LTR (57).

Retroviral *UBE2T* expression vectors

In order to demonstrate that *UBE2T* deficiency of *UBE2T*−/− HeLa cells generated through CRISPR/Cas9 was indeed responsible for the DNA repair defects, a retroviral vector was constructed that overexpressed the wild-type *UBE2T* cDNA. As the *UBE2T*−/− HeLa cells were already resistant to neomycin, puromycin and hygromycin, the cDNA of the blastidicin resistance gene was cloned from the pHAGE.CMV.EG*SLX4* vector (a kind gift of Agata Smogorzewska, Rockefeller University, NY, USA) and inserted into the retroviral pS91 UBE2T-IREs-puroR vector using NcoI-ClaI. The final retroviral vector expressed the blastidicin resistance gene after the IRES sequence. Infectious recombinant retroviral particles with the VSV-G phenotype were produced in 293T cells and used to transduce the HeLa cells at a multiplicity of infection of <0.2, as described previously (40).

RESULTS

Construction of dual fluorescence (DF) reporter constructs for the maternal *UBE2T* locus

The wild-type *UBE2T* allele in humans comprises seven exons with the translation start in exon 2, and two identical AluYa5 elements in the same orientation (www.ensembl.org). The first Alu element is located ∼180 bp 3′ of the exon1/intron 1 border, and a second Alu element is ∼180 bp 3′ of the exon 6/intron 6 border (Figure 1A). The mutant maternal allele contains a duplication of exons 2–6 and the mutant paternal allele exhibits a deletion of exons 2–6. Both mutant alleles seem to have occurred by Alu-mediated recombination. We have shown previously that the paternal allele is present as a founder mutation at low frequencies in the Italian and the German populations. However, we did not detect the maternal duplication in almost 2000 individuals (40) suggesting that it is much more restricted to the maternal lineage. In the proband, spontaneous deletion of the maternally duplicated exons 2–6 by recombination between...
Figure 2. DSB-induced recombination in DF cells. (A) I-Sce1 expression does not cause loss of either color marker gene in DF1 cells, but leads to preferential loss of eGFP expression in DF2 cells, and preferential loss of dTomato expression in DF3 cells. (B) Key to flow cytometry profiles. In the absence of I-Sce1 digestion cells contain dTomato (RFP+) and eGFP (GFP+) marker genes and appear in the upper right quadrant (DP, double positive). Loss of the eGFP gene renders cells red (R, upper left, RFP+, GFP-). Loss of the dTomato gene renders cells green (G, lower right, GFP+, RFP-). Loss of both marker genes renders cells double negative (DN, lower left, RFP-, GFP-). (C-E) Flow cytometry profiles of untreated (no I-Sce1) or I-Sce1 transfected (+I-Sce1) DF1, DF2, or DF3 cells, respectively. The data shown are representative of four or more experiments on each cell type. F, PCR analysis of control and I-Sce1 treated DF cell lines. •, I-Sce1-dependent aberrant recombination product.
Quantification of homology directed recombination events by flow cytometry

DF1, 2 and 3 cells were initially >95% double positive (DP) (RFP+, eGFP+) (Figure 2B–E). It is predicted that homologous recombination between proximal Alu elements would produce red DF2 cells by Alu 2/3 recombination and green DF3 cells by Alu 1/2 recombination, while recombination between distal Alu 1/3 elements would produce double negative cells. We observed that in vivo I-Sce1 expression did not significantly change the flow profile of DF1 cells which lack an I-Sce1 site (Figure 2C). However, representative results show that after I-Sce1 transfection, >30% of DF2 cells lost the eGFP marker (red, RFP+, eGFP–; upper left quadrant) (Figure 2D), and >30% of DF3 cells lost the dTomato marker (green, RFP–, eGFP+) (lower right quadrant) (Figure 2E). Approximately 10% of DF2 or DF3 cells lost both color markers (double negative (DN) RFP–, eGFP–; lower left quadrant), most probably due to recombination between the outermost Alu elements (Alu 1/3).

To confirm the extent of I-Sce1 induced deletions, we performed PCR across the ectopic integration site in each of the reporter cell lines. Lanes 1 and 2 (DF1 and DF1+I-Sce1) showed no deletions in the reporter construct in the DF1 cells upon I-Sce1 expression (Figure 2F), as anticipated. In contrast, I-Sce1 digested DF2 and DF3 cell DNAs displayed distinct lower molecular weight major bands (Figure 2F, lanes 4, 6) corresponding to deletion products expected of recombination between the proximal (Alu 1/2) and distal (Alu 2/3) Alu sites, consistent with the changed flow cytometry patterns of these cells after I-Sce1 expression. Specifically, recombination between the homologous Alu 1 and Alu 2 sites was found to generate a major PCR band of approximately 3.5 kb after deletion of the dTomato gene in RFP–, eGFP+ (green) DF2 cells (3.4% of total cells) and DF3 cells (30.2% of total cells).

Recombination between Alu 2 and Alu 3 generated a major PCR band of approximately 3.5 kb and deleted the eGFP gene in RFP+, eGFP– (red) DF2 cells (33.8% of total cells) and DF3 cells (1.1% of total cells). Recombination between Alu 1 and Alu 3 generated a major 1.4 kb PCR band and deleted both the dTomato and eGFP genes in RFP–, eGFP– (double negative) DF2 (9.7% of total cells) or DF3 cells (14.6% of total cells). Thus, in I-Sce1 treated DF2 or DF3 cells, more than ~45% of the cells underwent recombination (red, green, double negative cells).

As described in detail below, we estimated the percentage of DF2 double positive cells that had undergone recombination in vivo after I-Sce1 transfection, by in vitro I-Sce1 digestion of a full-length PCR product spanning the DF2 ectopic site. Since the DF2 full-length PCR product could only have come from double positive cells, and more than ~60% of this PCR product was resistant to in vitro I-Sce1 cleavage (Figure 4I), these results indicate that >75% of the ectopic sites (45% recombinant cells plus 60% of double positive cells (53.1% of total) had been cut by I-Sce1 in vivo and then further processed by DNA repair. Thus, Alu-mediated homology-directed recombination events had occurred in >30% of DF2 cells (red) and DF3 cells (green) following introduction of the I-Sce1 DSB within the reporter constructs. However, based on the resistance of the ectopic site PCR product to in vitro I-Sce1 digestion, additional repair mechanisms (e.g. NHEJ/MMEJ) were also active in >60% of cells where both fluorescent reporters were retained.

Single colony PCR characterization of recombinants

Because standard PCR on DNA from unsorted cultures may not disclose recombination products that arise from minor percentages of the total cell population, we took a single colony PCR approach to assess the fluctuation between cells following I-Sce1 digestion. Eight days after I-Sce1 plasmid transfection, DF2 cells were FACS sorted into individual double positive, red, green, and double negative cells and clonally expanded. Genomic DNA was harvested from randomly selected clones and amplified by PCR with primers across the ectopic site. In DNA from double positive (RFP+, eGFP+) DF2 cells, PCR across the single ectopic site (Figure 3A) generated a major band of ca. 5.7 kb in addition to bands of lesser intensity that presumably resulted from recombination during clonal expansion (Figure 3B). The majority of red (RFP+, eGFP–) cells displayed a major band of ca. 3.3 kb, consistent with recombination by sister chromatid HR or intrachromosomal SSA between the Alu 2 and Alu 3 elements flanking the I-Sce1 site (Figure 3C).

None of the colonies that turned green (RFP–, eGFP+) showed the ca. 3.3 kb band predicted by homology-directed repair between Alu 1 and Alu 2, but apparently had undergone a more complex series of recombinations generating smaller and larger PCR products that retained the eGFP gene and PCR primer binding sites (Figure 3D). Surprisingly, only a minority of the double negative (RFP–, eGFP–) cell clones showed the expected recombination between Alu 1 and Alu 3 (Figure 3E, lane 9).

The differences in the PCR profiles of the DNA from unsorted vs. sorted cells was striking. Following PCR of DNA from unsorted cells (Figure 2F), the most abundant cell population gave the expected ca. 3.5 kb PCR product, whereas these may still have comprised a minor percentage of the total cells. In contrast, in the PCR of DNA from sorted and cloned cells (Figure 3), even the lesser abundance green cells could give products that were different from the expected Alu 1/2 recombiant.

The data of Figures 2 and 3 argue that the most plausible mechanism of in vivo reversion of the maternally duplicated UBE2T gene resulted from a spontaneous double strand break that led to homology-directed recombination between Alu 2 and Alu 3 elements. Additionally, it is likely that a substantial percentage of breaks did not recombine by classical RAD51-dependent homologous recombination to restore the WT allele, but instead resulted in cells carrying alternative recombinations that were selected against in the patient’s hematopoietic compartment.

Role of DNA repair proteins in Alu-mediated recombination at the model UBE2T locus

To test the mechanism of recombination in the model UBE2T locus further, DF2 cells were exposed to I-Sce1 while proteins involved in nonhomologous end joining
Figure 3. PCR analysis of flow sorted DF2-derived cell clones detects homology directed and nonclassical recombination. DF2 cells were treated with I-SceI. Eight days later, DNA was isolated from each of thirty-six flow sorted colonies and analyzed by PCR. (A) map of the DF2 cell ectopic site construct; (B) RFP+, GFP+ cell clones; (C) RFP+, GFP– cell clones; (D) RFP–, eGFP+ cell clones; (E) RFP–, eGFP– cell clones.

(NHEJ) or HR were chemically inhibited or knocked down by siRNA. First, we treated I-SceI transfected DF2 cells with caffeine (Figure 4A), which preferentially inhibits the major DNA repair kinases ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and RAD3-related) (58–60). Compared to control cells digested with I-SceI (Figure 4B), caffeine significantly decreased the percentage of red, green, and double negative cells, and consequently increased the fraction of double positive cells (Figure 4C, H). This effect on recombination efficiency was more dramatic when using the ATM specific inhibitor KU60019 (Figure 4D, H), consistent with the stimulation of DSB end resection and downstream steps in HR and NHEJ by ATM (58–62).

The CtIP (C-terminal binding protein 1 interacting protein) nuclease catalyzes an early step in end resection of DNA DSBs in conjunction with the MRN complex, thereby terminating NHEJ and initiating HR (63–66). siRNA-mediated inhibition of CtIP (Figure 4E, H) or chemical inhibition of the RAD51 recombinase (RAD51i B02, Figure 4F, H; RAD51i RI-1, RAD51i RI-2, Supplementary Figure S3) also significantly decreased the homology-directed recombination that produced red cells upon I-SceI digestion. In contrast, specific inhibition of the NHEJ protein DNA-PKcs by NU7026 (67) did not significantly change the percentage of red cells (Figure 4G, H), as expected if RFP+, eGFP– cells resulted from HR or SSA. Inhibition of NHEJ by NU7026 decreased the percentage of double negative and green cells, but increased the percentage of double positive (yellow) cells, most likely by promoting precise religation of the I-SceI cut (68).

To distinguish between in vivo precise religation vs. mutagenic NHEJ of the cleaved I-SceI site, we carried out PCR across the ectopic site in DNA from DF2 cells transfected with I-SceI. We then digested the PCR product with I-SceI in vitro (Figure 4I). DF2 cells transfected with I-SceI and
Figure 4. Recombination in DF cells involves canonical HR and noncanonical pathways. (A) Map of the DF2 cell ectopic site construct. (B–G) Representative flow cytometric analyses of DF2 cells treated with I-Sce1. (B) Control siRNA; (C) caffeine; (D) ATM inhibitor KU60019; (E) left panel, siCtIP siRNA #1 + #2, right panel CtIP knockdown (western blot), •, crossreacting band; (F) RAD51 inhibitor BO2; (G) DNA-PKcs inhibitor NU7026. (H) Quantitation of flow cytometry results. Data are the means ± S.D. of three biological replicates each assayed in triplicate (paired Student’s t-test). Values of $P > 0.05$ were considered not significant (ns). I, In vitro I-Sce1 digestion of ectopic site PCR products from I-Sce1 transfected DF2 cells (B–G, see text for details). An enlargement of part of the DF2 cell ectopic site construct is shown to illustrate the PCR product (black bar) used for in vitro I-Sce1 digestion. Borders indicate that samples are from nonadjacent gel lanes.
treated with caffeine or ATM showed increased in vitro cutting of the ectopic site PCR product relative to cells treated with siControl (Figure 4I, lanes 1–3). Inasmuch as these cells displayed reduced homology-directed repair (i.e. red cells, Figure 4C, D, H), we conclude that the increased in vitro cutting of the PCR product reflected an increase in precise ligation in vivo. In contrast, the ratio of in vitro cut to uncut PCR product was reduced in DNA from cells treated with siCtIP or RAD51i (Figure 4E, F, H). Finally, inhibition of NHEJ by DNA-PKcsi resulted in an increase in the ratio of cut vs. uncut PCR product (Figure 4I, lane 6). Since DNA-PKcsi did not significantly change HDR (Figure 4H), we presume this increased cutting in vitro is due to enhanced precise ligation when DNA-PKcsi and NHEJ end processing are inhibited in vivo.

Based on the results of PCR in the FACS sorted cell clones (Figure 3D, E), we hypothesize that NHEJ was involved in the aberrant recombination occurring in the double negative (RFP–, eGFP–) and green (RFP–, eGFP+) I-Sce1 transfected DF2 cells. While the results of CtIP knockdown and RAD51i treatment are consistent with a reduction in HR, CtIP is also required for SSA (69), and knockdown of CtIP end resection can also be compensated by increased NHEJ (70,71), which would reduce in vitro I-Sce1 cutting. Although small-molecule RAD51 inhibitors have been shown to reduce HR (72–75), the possibility of off-target effects of these compounds remains.

Therefore, we sought an additional test of whether RAD51-dependent HR was responsible for contraction of the model UBE2T locus that produced red (RFP+, eGFP–) DF2 cells. As shown in Figure 5, we assayed the effects of knocking down BRCA2 (Figure 5A), which interacts with RAD51 and controls its translocation into the nucleus, nucleofilament formation and assembly of RAD51 foci in response to DNA damage (76–79). I-Sce1 was expressed in DF2 control cells or cells treated with BRCA2 siRNA (Figure 5B-F). The percentage of double positive (RFP+, eGFP+) cells increased significantly with BRCA2 knockdown (Figure 5F). By contrast, BRCA2 knockdown had no effect on the fraction of red cells produced by I-Sce1 expression. Instead, the percentage of green (RFP–, eGFP+) and double negative (RFP–, eGFP–) cells both decreased when BRCA2 was depleted. Conversely, the fraction of double positive cells was increased by compensating NHEJ/MMEJ, and mutagenic recombinations which produce green and double negative cells (Figure 3) were also enhanced. Thus, the change in the percentages of double positive, double negative and green cells indicates the biological effectiveness of the BRCA2 knockdown, while the absence of an effect on the percentage of red cells argues that BRCA2-independent SSA is responsible for the DF2 Alu-mediated contraction, and that downregulation of HR by BRCA2 depletion promotes alternative, mutagenic forms of recombination (80–83).

**Cis-acting effects of the Alu 1/IVS1 sequence on recombination at the model UBE2T locus**

Digestion of DF2 cells with I-Sce1 resulted in the expected 1.4 kb Alu 1/3 recombination product when DNA from unsorted cells was analyzed (Figure 2F), however, only about 9% of the cells fell in the double negative population (Figure 2D) and only 1 of 9 randomly selected double negative cell clones produced the 1.4 kb PCR product predicted to arise from Alu1/3 recombination (Figure 3D). To determine if Alu 1/IVS1 was exerting an effect in cis on homology directed recombination between Alu2/3, two additional cell lines, DF4 and DF5, were constructed that deleted Alu 1/IVS1 (Figure 6A). The DF4 cell line differs from DF5 in that DF4 does not contain an I-Sce1 site; consequently, I-Sce1 transfection did not change the flow cytometry profile of DF4 cells (Figure 6B). I-Sce1 digestion of DF2 or DF5 cells resulted in similar percentages of double positive cells (Figure 6C, D). Surprisingly, I-Sce1 expression in DF5 cells (Figure 6C) produced approximately half as many red (RFP+, eGFP–) cells and twice as many double negative (RFP–, eGFP–) recombinants as in I-Sce1 digested DF2 cells (Figure 6D). This result indicates that Alu 1/IVS1 acts in cis to promote Alu 2/3 homology-directed recombination to yield red cells, and to suppress recombination leading to double negative cells.

The decrease in the percentage of DF5 red cells after I-Sce1 transfection is not due to reduced efficiency of I-Sce1 digestion, as the total percentage of red, green and double negative cells was closely similar between I-Sce1 digested DF2 and DF5 cells, but the ratio of green:red:double negative recombinant cells changed from approximately 1:10:2.5 in DF2 cells to approximately 1:2:3 in DF5 cells. To analyze the I-Sce1 induced deletions, we performed PCR across the ectopic site in DF4 and DF5 cells. The gel of Figure 6F confirmed that I-Sce1 digested DF5 cells displayed a lower molecular weight band of ~3.1 kb corresponding to the deletion product expected for recombination at the Alu 2 and Alu 3 sites, consistent with the flow cytometry patterns of these cells (Figure 6C, D; RFP+, eGFP–).

We also observed that ~22% of the I-Sce1 digested DF5 cells were double negative (RFP–, eGFP–). Taken with the PCR results on double negative cell DNA in Figure 3C, we judged that amplified PCR products smaller than 3.1 kb retained the PCR primer binding sites and were the result of excessive nuclease digestion at the I-Sce1 break or spontaneous breakage and recombination at sites other than the Alu 2 and Alu 3 repeats.

**Effect of UBE2T knockout on homology directed recombination**

The conclusion that a recombination event with generation of a wild-type UBE2T allele in the FANCT patient is the direct consequence of homology directed recombination (HDR) and the reason for the phenotypic reversion in a hematopoietic cell containing the inherited partial duplication of the UBE2T gene, is based on the supposition that FANCT/UBE2T deficient cells can still perform HDR. Because several FA proteins, including UBE2T, have been implicated in multiple forms of DNA repair including homology directed recombination (26,84,85), we wished to test directly the effect of UBE2T loss on HDR efficiency. Thus, lentivirus-mediated CRISPR-Cas9 was used to knockout the UBE2T genes in DF3 cells (UBE2TΔ3 cells) (Figure 7A) and then single cell clones were expanded. Homology directed repair of the I-Sce1 cut in DF3 cells (Figure 7B) is
Figure 5. BRCA2 knockdown does not affect Alu-mediated SSA, but enhances end joining and mutagenic recombination. (A) Western blot of whole cell extracts of DF2 cells treated with siControl or siBRCA2. (B) Flow cytometry profile of DF2 cells treated with control siRNA. (C) Flow cytometry profile of DF2 cells treated with control siRNA and I-Sce1. (D) Flow cytometry profile of DF2 cells treated with BRCA2 siRNA. (E) Flow cytometry profile of DF2 cells treated with BRCA2 siRNA and I-Sce1. (F) Quantitation of flow cytometry results. Values are means ± S.D. of three biological replicates each assayed in triplicate (paired Student’s t-test). P values compare I-Sce1 treated samples ± BRCA2 siRNA.
Figure 6. *Cis*-acting effects of the Alu1/IVS1 sequence on recombination at the model UBE2T locus. (A) DF4 cells contain the same genomic integrant as DF1 cells except for deletion of the first AluYa5 element and IVS1 fragment. DF5 cells contain the same integrant as DF4 cells, but also contain an I-Sce1 site upstream of the eGFP gene (as in DF2 cells). (B–D) Flow cytometry profile of DF4, DF2 or DF5 cells, respectively, either untreated or transfected with I-Sce1. (E) Quantitation of flow cytometry results. DP, double positive; R, red; G, green; DN, double negative. Data are the means ± S.D. of three biological replicates each assayed in triplicate (paired Student’s *t*-test). Values of *P* > 0.05 were considered not significant (ns). F, PCR analysis of control and I-Sce1 treated DF4 and DF5 cell lines. •, I-Sce1-dependent aberrant recombination product.
Figure 7. UBE2T knockout partially inhibits HR. (A) CRISPR-Cas9 knockout of UBE2T in DF3 and U2OS cells (western blot). Note the greater abundance of UBE2T in tetraploid DF3 cells. (B) Schematic of the DF3 cell line construct. (C) Representative flow cytometry profiles of DF3 control cells and UBE2T CRISPR knockout DF3 cells with or without I-Sce1 digestion. (D) Quantitation of flow cytometry profiles of triplicate biological replicates assayed in triplicate. Values are the means ± S.D. (paired Student’s t-test). KO, CRISPR knockout DF3 cells; UBE2T+, KO cells reconstituted with lentiviral UBE2T cDNA. (E) DR-GFP schematic. (F) Quantitation of homology directed repair in U2OS control cells and UBE2T−/− CRISPR knockout (KO) U2OS cells. Naïve, unedited U2OS cells; KO, CRISPR knockout of UBE2T; KO + EV, KO cells treated with lentivirus empty vector; KO + UBE2T, KO cells reconstituted with lentiviral UBE2T cDNA; LC, cells treated with control lentiviral vector. Values are means ± S.D. of three biological replicates each assayed in triplicate (paired Student’s t-test).
predicted to yield green cells (RFP−, GFP +; Figure 7C). To assess the efficiency of homologous recombination we compared the levels of green cells produced when naïve DF3 cells or UBE2TΔΔ DF3 cells were transiently transfected with the I-Sce1 vector. Compared to naïve cells, UBE2TΔΔ cells showed an approximate 50% decrease in the percentage of RFP−, eGFP+ (green) cells (Figure 7C, D). We conclude that significant homology-directed recombination could still occur at the Alu repeats in the model UBE2T allele of UBE2TΔΔ cells when a DSB breaks occurred between flanking Alu repeats.

For comparison, the UBE2T genes were also knocked out in U2OS cells using CRISPR-Cas9 lentivirus (Figure 7A). The UBE2TΔ−/− knock-out U2OS cells contain the direct repeat (DR-GFP) construct (Figure 7E), which has been used widely as a reporter for HR (86–91). In these cells, the upstream SceGFP gene is inactive due to the presence of a stop codon within the I-Sce1 cleavage site, while a second partial GFP fragment of 812 bp (iGFP) is present 3.7 kb downstream on the same chromosome. I-Sce1 cleavage of this construct can lead to a homology-dependent gene conversion event between the iGFP and the broken SceGFP sequence, and thereby restore a functional GFP protein which can be measured by flow cytometry.

Biallelic CRISPR-Cas9 knockout of the U2OS cell UBE2T genes (UBE2TΔ−/−) resulted in a significant decrease in GFP + cells after I-Sce1 cleavage compared to naïve control U2OS cells or the same U2OS UBE2TΔ−/− cells corrected with a viral UBE2T cDNA expression vector (KO + UBE2T; Figure 7F). These results showed that the UBE2T protein contributes to the efficiency of homology dependent repair, however, it is not absolutely required for HR repair to occur. The observation that UBE2TΔ−/− cells retained more than half of the HR activity of UBE2TΔ+/Δ cells implies that redundant pathways to homology directed repair are active in these engineered FA-T cells.

**DISCUSSION**

The E2 ubiquitin conjugases UBE2T and UBE2W can act in the ubiquitination of FANCI/D2. However, UBE2T is only the conjugase required for activation of the FA pathway in response to MMC/ICL, and biallelic loss-of-function mutations of UBE2T lead to the FA phenotype of chromosome instability (36–39,92–94). Our experiments to model recombination at the UBE2T locus were prompted by an FA patient with inactivating duplication and deletion mutations in UBE2T and whose fibroblasts were hypersensitive to the genotoxins DEB and cisplatin, and defective in monoubiquitination of FANCD2 in response to MMC (40). Surprisingly, the UBE2T duplication was almost completely absent in genomic DNA from the patient’s peripheral blood, consistent with normal thrombocyte, leukocyte and platelet counts as well as normal bone marrow cellularity.

To test whether homology dependent recombination at the UBE2T AluYa5 elements could account for the genetic reversion in this patient, we integrated single copy model UBE2T alleles into HeLa/406 cells using FLP recombinase (45–50). The model UBE2T alleles contained red (dTomato) and green (eGFP) reporter genes separated by an I-Sce1 cleavage site so that the recombination events after DNA double strand breakage could be monitored on protein levels by flow cytometry.

I-Sce1 expression in the reporter cell lines DF2 and DF3 resulted in the cleavage of greater than 75% of the model UBE2T alleles and changes in color that were monitored by fluorescence microscopy and flow cytometry. The loss of the dTomato or eGFP ORFs and the sizes of the major PCR products from the I-Sce1 treated cells were consistent with homology-dependent Alu-mediated recombination. From these results, we conclude that an endogenous DSB likely initiated recombinational reversion of the duplicated maternal UBE2T allele in the proband and possibly partial deletion of one UBE2T allele in the father.

The structures of the recombined reporter loci indicated that HDR had occurred primarily between pairs of Alu elements. However, analysis of flow sorted clonal cell populations indicated that more complex mechanisms leading to unexpected patterns of loss of both color reporter genes were also active in a minority (<15%) of cells. Knockdown or inhibition of proteins involved in DNA damage signaling and HR (RAD51, ATM, CtIP), but not NHEJ (DNA-PKcs), inhibited recombination between Alu elements, supporting the view that classical single strand annealing was responsible for Alu-mediated recombination. In DF2 cells, inhibition shifted approximately half of the recombinations from HDR (RFP+, eGFP−) to NHEJ to yield RFP+ eGFP+ cells that generated I-Sce1 resistant PCR products.

Single strand annealing is a RAD51-independent mechanism of recombination (42,80,81,95). To test for the role of RAD51 in I-Sce1 repair in the model UBE2T locus, we used the RAD51 inhibitors B02, RI-1 and RI-2, which have been used extensively to analyze RAD51-mediated recombination (72–75,96–115). Each of these drugs reduced Alu-mediated HDR by ~50%; however, these drugs may have off-target effects in addition to inhibition of RAD51. Therefore, we also tested the DNA repair in conjunction with BRCA2 knockdown, which has been shown to block RAD51-dependent HR, and increase error-prone forms of recombination (81). Our results are consistent with these previous observations, in that BRCA2 knockdown increased the percentage of RFP−, GFP+ and RFP−, eGFP− cells, which result from aberrant recombination (Figure 3). However, BRCA2 knockdown did not affect the percentage of RFP+ eGFP− cells, the majority of which appear to have resulted from SSA (Figure 5). Although not all functions of RAD51 (e.g. S-phase focus formation (116) and replication fork reversal (117)) are dependent on BRCA2 (118), we conclude that RAD51-independent homology directed SSA is responsible for at least a large proportion of Alu-mediated recombination in our system.

We also observed that Alu 1/IVS1 acts in cis to promote Alu 2/3 homology directed recombination to yield red cells. In DF2 cells, Alu 1 is 2 kb upstream of Alu 2, which is well within the length of DNA that is resected in advance of homology directed repair (119,120). In flow cytometry-
sorted cells (Figure 3), only one of nine double negative clones showed the expected Alu 1/3 recombination product. This result suggests that there are sequences in the construct that allow homologous recombinations that retain (lanes 3, 6) or delete (lanes 1, 2, 4, 5, 7, 8) the PCR primer sites. One possible explanation for the positive influence of Alu 1/IVS1 on HR is that the Alu 1 sequence or its binding proteins (121–123) on the sister chromatid aid in positioning the Alu 2/IVS6 to anneal to the Alu3/IVS6 sequence during intrachromosomal SSA, akin to the phenomenon of transvection (124–127).

Since HR had been implicated in the reversion of the duplication in the UBE2T−/− patient’s hematopoietic cells, we tested for this activity following UBE2T knockout in DF3 and U2OS cells. An approximate 50% decrease in RFP-eGFP+ cells in UBE2T knockout DF3 cells implied that redundant homology-dependent repair pathways are operable for Alu-mediated recombination. In UBE2T−/− U2OS cells, the DR-GFP assay showed a reproducible decrease of ~40% in HR, suggesting that UBE2T plays a role in HR in addition to activation of FANCI/D2 for ICL removal, but that one or more UBE2T-independent parallel pathways also exist for residual HR in UBE2T−/− cells. An overview of the decrease in homologous recombination detected by the DR-GFP assay due to insufficiency of several FA or HR-related proteins (Supplementary Table S1) shows a wide range of effects, with knockdown of several known FA proteins showing incomplete inhibition of HR, as in the case of UBE2T knockout.

We propose that UBE2T-dependent homology directed recombination is one mechanism of Alu-mediated reversion of the model UBE2T locus and that in UBE2T null patient cells, a UBE2T-independent residual mechanism of HR such as SSA was responsible for contraction of the partially duplicated UBE2T locus. We conclude that a spontaneous DSB in the duplicated UBE2T locus of the FANC-T patient that had occurred in an hematopoietic stem cell was sufficient for UBE2T independent, Alu-mediated recombination that restored a wild-type UBE2T gene and thereby provided survival advantage for that stem cell and its progeny.

Finally, we note that the dual fluorescence flow cytometric assay for recombination in DF cells is quantitatively responsive to DNA double strand breaks and the manipulation of DNA repair pathways, indicating that this is a robust gateway system that could be adapted to the analysis of Alu-mediated homologous recombination in the human BRCA1 locus in breast tumors (6) and other diseases including FA (9,128), or more generally to probe the causes and consequences of DSBs in human cells (129). Combined with knockdowns of specific genes of interest or testing of chemical/medicinal compounds, the dual fluorescence system can quantitatively report on the contribution of specific proteins to HR and NHEJ and is therefore well suited for high throughput systematic studies of Alu-mediated recombination events.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors wish to thank John Turchi, David Hitch and S. Dean Rider for insightful comments on the manuscript.

FUNDING

National Institutes of Health (NIH) [GM099874, GM122976 to M.L., CA155294 to H.H.]; Lilly Foundation Physician/Scientist Initiative [to H.H.]; Walther Cancer Foundation [to H.H.]; Department of Defense [DoD W81XWH-18-1-0269 to P.A.]; WSU Biomedical Sciences Ph.D. Program [to T.W.L., J.B.].

Conflict of interest statement. None declared.

REFERENCES

1. Lander, E. et al. (2001) Initial sequencing and analysis of the human genome. Nature, 409, 860–921.
2. Elliott, B., Richardson, C. and Jasin, M. (2005) Chromosomal translocation mechanisms at intronic alu elements in mammalian cells. Mol. Cell, 17, 885–894.
3. Deininger, P.L. and Batzer, M.A. (1999) Alu repeats and human disease. Mol. Genet. Metab., 67, 183–193.
4. Batzer, M.A. and Deininger, P.L. (2002) Alu repeats and human genomic diversity. Nat. Rev. Genet., 3, 370–377.
5. Levran, O., Doggett, N.A. and Auerbach, A.D. (1998) Identification of Alu-mediated deletions in the Fanconi anemia gene FANCA. Hum. Mutat., 12, 145–152.
6. Rohloff, E.M., Puget, N., Graham, M.L., Weber, B.L., Garber, J.E., Skrzynia, C., Halperin, J.L., Lenoir, G.M., Silverman, L.M. and Mazoyer, S. (2000) An Alu-mediated 7.1 kb deletion of BRCA1 exons 8 and 9 in breast and ovarian cancer families that results in alternative splicing of exon 10. Genes Chromosomes Cancer, 28, 300–307.
7. Rothberg, P.G., Ponnuru, S., Baker, D., Bradley, J.F., Freeman, A.I., Cibis, G.W., Harris, D.J. and Herut, D.P. (1997) A deletion polymorphism due to Alu-Alu recombination in intron 2 of the retinoblastoma gene: association with human gliomas. Mol. Carcinog., 19, 69–73.
8. Mizunuma, M., Fujimori, S., Ogino, H., Ueno, T., Inoue, H. and Kamatani, N. (2001) A recurrent large Alu-mediated deletion in the hypoxanthenine phosphoribosyltransferase (HPRT1) gene associated with Lesch–Nyhan syndrome. Hum. Mutat., 18, 435–443.
9. Kim, S., Cho, C.S., Han, K. and Lee, J. (2016) Structural variation of alu element and human disease. Genomics Inform., 14, 70–77.
10. Kutler, D.I., Singh, B., Satagopan, J., Batish, S.D., Berwick, M., Giampietro, P.F., Hanenberg, H. and Auerbach, A.D. (2003) A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood, 101, 1249–1256.
11. Park, J.Y., Virts, E.L., Jankowska, A., Wick, C., Othman, M., Chakraborty, S.C., Vance, G.H., Alkuraya, F.S., Hanenberg, H. and Andreassen, P.R. (2016) Complementation of hypersensitivity to DNA interstrand crosslinking agents demonstrates that XRCC2 is a Fanconi anemia gene. J. Med. Genet., 53, 672–680.
12. Bluteau, D., Masliah-Planchon, J., Clairmont, C., Rousseau, A., Cacciardi, R., Dubois d’Enghien, C., Bluteau, O., Cuccuini, W., Gachet, S., Peffault de Latour, R. et al. (2016) Biallelic inactivation of REV7 is associated with Fanconi anemia. J. Clin. Invest., 126, 3580–3584.
13. Mamrak, N.E., Shimamura, A. and Howlett, N.G. (2017) Recent discoveries in the molecular pathogenesis of the inherited bone marrow failure syndromes. Blood Rev., 31, 93–99.
14. Inano, S., Sato, K., Katsuki, Y., Kobayashi, W., Tanaka, H., Nakajima, K., Nakada, S., Miyoshi, H., Knies, K., Takaori-Kondo, A. et al. (2017) RFWD3-Mediated ubiquitination promotes timely removal of both RPA and RAD51 from DNA damage sites to facilitate homologous recombination. Mol. Cell, 66, 622–634.
15. Feeney, L., Munion, J.M., Lachaud, C., Toth, R., Appleton, P.L., Schindler, D. and Rouse, J. (2017) RPA-mediated recruitment of the E3 ligase RFWD3 is vital for interstrand crosslink repair and human health. Mol. Cell, 66, 610–621.
16. Maarek,O., Jonveaux,P., Le Coniat,M., Derre,J. and Berger,R. (1996) Fanconi anemia and bone marrow clonal chromosome abnormalities. *Leukemia*, 10, 1700–1704.

17. Schlacher,K., Wu,H. and Jasin,M. (2012) A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1. *Cancer Cell*, 22, 106–116.

18. Auerbach,A. (2009) Fanconi anemia and its diagnosis. *Mutat. Res.*, 668, 4–10.

19. Barthelemy,J., Hanenberg,H. and Leffak,M. (2016) FANCJ is essential to maintain microsatellite structure genome-wide during replication stress. *Nucleic Acids Res.*, 44, 6803–6816.

20. Langevin,F., Crossan,G.P., Rosado,I.V., Arends,M.J. and Patel,K.J. (2015) FANCJ contributes to the toxic effects of naturally produced aldehydes in mice. *Nature*, 475, 53–58.

21. Garcia-Rubio,M.L., Perez-Calero,C., Barroso,S.I., Tumini,E., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2015) The Fanconi anemia pathway protects genome integrity from R-loops. *PLoS Genet.*, 11, e1005674.

22. Kais,Z., Rondinelli,B., Holmes,A., O’Leary,C., Kozono,D., Sumpter,R. Jr., Sirasanagandla,S., Fernandez,A.F., Wei,Y., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2013) FANCI to promote replication fork recovery. *Cell Biol. Int.*, 37, 867–836.

23. Kais,Z., Rondinelli,B., Holmes,A., O’Leary,C., Kozono,D., Sumpter,R. Jr., Sirasanagandla,S., Fernandez,A.F., Wei,Y., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2013) FANCI to promote replication fork recovery. *Cell Biol. Int.*, 37, 867–836.

24. Kais,Z., Rondinelli,B., Holmes,A., O’Leary,C., Kozono,D., Sumpter,R. Jr., Sirasanagandla,S., Fernandez,A.F., Wei,Y., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2013) FANCI to promote replication fork recovery. *Cell Biol. Int.*, 37, 867–836.

25. Kais,Z., Rondinelli,B., Holmes,A., O’Leary,C., Kozono,D., Sumpter,R. Jr., Sirasanagandla,S., Fernandez,A.F., Wei,Y., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2013) FANCI to promote replication fork recovery. *Cell Biol. Int.*, 37, 867–836.

26. Kais,Z., Rondinelli,B., Holmes,A., O’Leary,C., Kozono,D., Sumpter,R. Jr., Sirasanagandla,S., Fernandez,A.F., Wei,Y., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2013) FANCI to promote replication fork recovery. *Cell Biol. Int.*, 37, 867–836.

27. Kais,Z., Rondinelli,B., Holmes,A., O’Leary,C., Kozono,D., Sumpter,R. Jr., Sirasanagandla,S., Fernandez,A.F., Wei,Y., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2013) FANCI to promote replication fork recovery. *Cell Biol. Int.*, 37, 867–836.

28. Kais,Z., Rondinelli,B., Holmes,A., O’Leary,C., Kozono,D., Sumpter,R. Jr., Sirasanagandla,S., Fernandez,A.F., Wei,Y., Herrera-Moyano,E., Rosado,I.V. and Aguilera,A. (2013) FANCI to promote replication fork recovery. *Cell Biol. Int.*, 37, 867–836.
56. Flynn,E.K., Kamar,A., Lach,F.P., Donovan,F.X., Kimble,D.C., Narisu,N., Sanborn,E., Boulad,F., Davies,S.M., Gligio,A.P. 3rd et al. (2014) Comprehensive analysis of pathogenic deletion variants in Fanconi anemia genes. *Hum. Mutat.*, 35, 1342–1353.

57. Roellecke,K., Virtis,E.L., Einholz,R., Edson,K.Z., Altavera,B., Rossig,C., von Laer,D., Schekenbach,K., Wagenmann,M., Reinhardt,D. et al. (2016) Optimized human CYP4B1 in combination with the alkylator prodrug 4-impenoanol serves as a novel suicide gene system for adoptive T-cell therapies. *Gene Ther.*, 23, 615–626.

58. Cortez,D., Wang,Y., Qin,J. and Elledge,S.J. (1999) Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science*, 286, 1162–1166.

59. Bakr,A., Oing,C., Kocher,S., Borgmann,K., Dornreiter,I., Blackford,A.N. and Jackson,S.P. (2017) ATM, ATR, and DNA-PK: Response to double-strand breaks. *Cell*, 170, 1232–1247.

60. Doerks,T., Copley,R.R., Schultz,J., Ponting,C.P. and Bork,P. (2002) Systematic identification of novel protein domain families associated with nuclear functions. *Genome Res.*, 12, 47–56.

61. Makharashvili,N., Tubbs,A.T., Yang,S.H., Wang,H., Barton,O., Zhou,Y., Deshpande,R.A., Lee,J.H., Lobrich,M., Sleckman,B.P. et al. (2014) Catalytic and noncatalytic roles of the CtIP endonuclease in double-strand break end resection. *Mol. Cell.*, 54, 1022–1033.

62. Wang,H., Li,Y., Triung,L.N., Shi,L.Z., Hwang,P.Y., He,J., Do,J., Cho,M.J., Li,H., Negret,A. et al. (2014) CtIP maintains stability at common fragile sites and inverted repeats by end resection-independent endonuclease activity. *Mol. Cell.*, 54, 1012–1021.

63. Anand,R., Ranjha,L., Cannavo,E. and Cejka,P. (2016) Phosphorylated CtIP Functions as a Co-factor of the MRE11-RAD50-NBS1 Endonuclease in DNA End Resection. *Mol. Cell.*, 64, 940–950.

64. Makharashvili,N. and Paull,T.T. (2015) CtIP: A DNA damage response protein at the intersection of DNA metabolism. *DNA Repair (Amst.),* 32, 75–81.

65. Veuger,S.J., Curtin,N.J., Richardson,C.J., Smith,G.C. and Durkacz,B.W. (2003) Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1. *Cancer Res.*, 63, 6008–6015.

66. Lin,Y., Lukacsovich,T. and Waldman,A.S. (1999) Multiple pathways for repair of DNA double-strand breaks in mammalian chromosomes. *Mol. Cell Biol.*, 19, 8353–8360.

67. Yun,M.H. and Hiom,K. (2009) CtIP-RBCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature*, 459, 460–463.

68. Fouquain,A., Guenouill-Barbat,J., Lopez,B., Hall,J., Amor-Gueret,M. and Pennaneach,V. (2017) PARP2 controls double-strand break repair pathway choice by limiting 53BP1 accumulation at DNA damage sites and promoting end-resection. *Nucleic Acids Res.*, 45, 12325–12339.

69. Salles,D., Mencalha,A.L., Ireno,I.C., Wiesmuller,L. and Abdelhay,E. (2011) BCR-ABL stimulates mutagenic homologous DNA double-strand break repair via the DNA-end-processing factor CtIP. *Carcinogenesis*, 32, 27–34.

70. Huang,F., Motlekar,N.A., Burgwin,C.M., Napper,A.D., Diamond,S.L. and Mazin,A.V. (2011) Identification of specific inhibitors of human RAD51 recombinase using high-throughput screening. *ACS Chem. Biol.*, 6, 626–635.

71. Budke,B., Logan,H.L., Kalin,J.H., Zelivianskia,kas,A.S., Cameron McGuire,W., Miller,L.L., Stark,J.M., Kozikowski,A.P., Bishop,D.K. and Connell,P.P. (2012) RI-1: a chemical inhibitor of RAD51 that disrupts homologous recombination in human cells. *Nucleic Acids Res.*, 40, 7347–7357.

72. Berte,N., Piec-Staffa,A., Picche,N., Wang,M., Borgmann,K., Kaina,B. and Nikolova,T. (2016) Targeting homologous recombination by pharmacological inhibitors enhances the killing response of glioblastoma cells treated with alkylating drugs. *Mol. Cancer Ther.*, 15, 2665–2678.

73. Chen,Q., Cai,D., Li,M. and Wu,X. (2017) The homologous recombination protein RAD51 is a promising therapeutic target for cervical carcinoma. *Oncol. Rep.*, 38, 767–774.

74. Davies,A.A., Masson,J.Y., McIwraith,M.J., Stasiak,A.Z., Stasiak,A., Venkitaraman,A.R. and West,S.C. (2001) Role of BRCA2 in control of the RAD51 recombinase and DNA repair protein. *Mol. Cell.*, 7, 273–282.

75. Pellegrini,L., Yu,D.S., Lo,T., Anand,S., Lee,M., Blundell,T.L. and Venkitaraman,A.R. (2002) Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature*, 420, 287–293.

76. Shi,D.S., Pellegrini,L., Daniels,D.S., Yelenet,B., Craig,L., Bates,D., Yu,D.S., Shivi,i,M.K., Hitomi,C., Arvai,A.S. et al. (2003) Full-length archaeal RAD51 structure and mutants: mechanisms for RAD51 assembly and control by BRCA2. *EMBO J.*, 22, 4566–4576.

77. Anand,R., Ranjha,L., Cannavo,E. and Cejka,P. (2016) Phosphorylated CtIP Functions as a Co-factor of the MRE11-RAD50-NBS1 Endonuclease in DNA End Resection. *Mol. Cell.*, 64, 940–950.

78. Neugschwandtner,W., Siegmund,T. and Zabel,T. (2015) CtIP: A DNA damage response protein at the intersection of DNA metabolism. *DNA Repair (Amst.),* 32, 75–81.

79. Veuger,S.J., Curtin,N.J., Richardson,C.J., Smith,G.C. and Durkacz,B.W. (2003) Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1. *Cancer Res.*, 63, 6008–6015.

80. Lin,Y., Lukacsovich,T. and Waldman,A.S. (1999) Multiple pathways for repair of DNA double-strand breaks in mammalian chromosomes. *Mol. Cell Biol.*, 19, 8353–8360.

81. Berte,N., Piec-Staffa,A., Picche,N., Wang,M., Borgmann,K., Kaina,B. and Nikolova,T. (2016) Targeting homologous recombination by pharmacological inhibitors enhances the killing response of glioblastoma cells treated with alkylating drugs. *Mol. Cancer Ther.*, 15, 2665–2678.

82. Chen,Q., Cai,D., Li,M. and Wu,X. (2017) The homologous recombination protein RAD51 is a promising therapeutic target for cervical carcinoma. *Oncol. Rep.*, 38, 767–774.

83. Davies,A.A., Masson,J.Y., McIwraith,M.J., Stasiak,A.Z., Stasiak,A., Venkitaraman,A.R. and West,S.C. (2001) Role of BRCA2 in control of the RAD51 recombinase and DNA repair protein. *Mol. Cell.*, 7, 273–282.

84. Pellegrini,L., Yu,D.S., Lo,T., Anand,S., Lee,M., Blundell,T.L. and Venkitaraman,A.R. (2002) Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature*, 420, 287–293.

85. Shi,D.S., Pellegrini,L., Daniels,D.S., Yelenet,B., Craig,L., Bates,D., Yu,D.S., Shivi,i,M.K., Hitomi,C., Arvai,A.S. et al. (2003) Full-length archaeal RAD51 structure and mutants: mechanisms for RAD51 assembly and control by BRCA2. *EMBO J.*, 22, 4566–4576.

86. Anand,R., Ranjha,L., Cannavo,E. and Cejka,P. (2016) Phosphorylated CtIP Functions as a Co-factor of the MRE11-RAD50-NBS1 Endonuclease in DNA End Resection. *Mol. Cell.*, 64, 940–950.

87. Neugschwandtner,W., Siegmund,T. and Zabel,T. (2015) CtIP: A DNA damage response protein at the intersection of DNA metabolism. *DNA Repair (Amst.),* 32, 75–81.

88. Veuger,S.J., Curtin,N.J., Richardson,C.J., Smith,G.C. and Durkacz,B.W. (2003) Radiosensitization and DNA repair inhibition by the combined use of novel inhibitors of DNA-dependent protein kinase and poly(ADP-ribose) polymerase-1. *Cancer Res.*, 63, 6008–6015.

89. Lin,Y., Lukacsovich,T. and Waldman,A.S. (1999) Multiple pathways for repair of DNA double-strand breaks in mammalian chromosomes. *Mol. Cell Biol.*, 19, 8353–8360.

90. Berte,N., Piec-Staffa,A., Picche,N., Wang,M., Borgmann,K., Kaina,B. and Nikolova,T. (2016) Targeting homologous recombination by pharmacological inhibitors enhances the killing response of glioblastoma cells treated with alkylating drugs. *Mol. Cancer Ther.*, 15, 2665–2678.

91. Chen,Q., Cai,D., Li,M. and Wu,X. (2017) The homologous recombination protein RAD51 is a promising therapeutic target for cervical carcinoma. *Oncol. Rep.*, 38, 767–774.
