complex, giving local pulses of dNTPs at the appropriate times.

Such an S-phase chromosome feedback system could also explain temporal programming and modulation of S-phase length. Mec1 may act in cis to promote replication fork progression and in trans to inhibit late replication origin firing as well as S-phase exit. If Mec1-mediated fork progression were accompanied by coordinate alleviation of the two trans inhibitory effects, orderly progression of replication and post-fork development throughout the genome could be ensured; once all forks are resolved, the cell would exit S phase. Moreover, the length of S phase could be expanded or contracted as an integral process without altering either the number or the pattern of fired origins or the intrinsic rate of fork progression. Instead, the length of S phase could be governed by the rate of post-fork chromosome morphogenesis. Correspondingly, meiotic S phase appears to be an expanded version of mitotic S phase, and its length is modulated positively by meiotic cohesin Rec8 and negatively by meiotic pairing/recombination protein Spo11 (*28*). This model also explains why Mec1 (via Rad53) is a negative regulator of late-origin firing in unchallenged cells (*8*) and predicts that a *rad53* mutant should exhibit longer than normal S phase despite its early firing of late origins because of its inability to promote fork progression through *RSZs*. We observe exactly this effect in both mitosis and meiosis (supporting online text).

Throughout the mitotic and meiotic programs, chromosomal changes must occur in a regulated way throughout the genome, different chromosomal events must be coordinated with one another, and progression of the cell cycle must be linked to proper completion of such processes. We suggest that ATR/ATM-family proteins mediate such effects, not only during S phase but also potentially during G_1 (*5*), meiotic prophase (*6*, *7*), and in the cytoplasm, where ATM mediates signal transduction during normal cellular responses (*29*). Failure to carry out certain of these basic roles then contributes to the defects observed in the corresponding mutants in the absence of exogenous insult. By this view, DNA damage/replication checkpoint responses could represent specialized amplifications and applications of functions having more basic roles.

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Supporting Online Material

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Biallelic Inactivation of *BRCA2* in Fanconi Anemia

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Fanconi anemia (FA) is a rare autosomal recessive cancer susceptibility disorder characterized by cellular hypersensitivity to mitomycin C (MMC). Six FA genes have been cloned, but the gene or genes corresponding to FA subtypes B and D1 remain unidentified. Here we show that cell lines derived from FA-B and FA-D1 patients have biallelic mutations in *BRCA2* and express truncated BRCA2 proteins. Functional complementation of FA-D1 fibroblasts with wild-type *BRCA2* complementary DNA restores MMC resistance. Our results link the six cloned FA genes with *BRCA1* and *BRCA2* in a common pathway. Germ-line mutation of genes in this pathway may result in cancer risks similar to those observed in families with *BRCA1* or *BRCA2* mutations.

Fanconi anemia (FA) is a rare autosomal recessive cancer susceptibility syndrome characterized by congenital abnormalities, progressive bone marrow failure, and cellular hypersensitivity to DNA cross-linking agents, such as MMC and cisplatin (*1*, *2*). FA patients often develop acute myeloid leukemia (AML), but also develop squamous cell carcinomas, frequently of the head and neck or of the gynecologic system (*3*). Whether heterozygote carriers of FA gene mutations have an increased cancer risk remains unknown (*4*).

At least eight distinct complementation groups of FA (A, B, C, D1, D2, E, F, G) have been defined by somatic cell fusion studies (*5*–*7*), and six FA genes have been cloned (A, C, D2, E, F, G). The six known FA proteins interact in a common pathway (*8*). Five of the FA proteins (A, C, E, F, G) assemble in a multisubunit nuclear complex. Either in response to DNA damage (*8*) or during S phase of the cell cycle (*9*), this complex activates the monoubiquitination of the downstream D2 protein, thereby targeting D2 to BRCA1-containing nuclear foci. Biallelic mutation of an upstream FA gene disrupts the monoubiquitination of FANCD2, resulting in loss of

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FANCD2 foci and hypersensitivity to MMC.

Recent studies have suggested genetic interactions among the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, and the FA genes. First, disruption of *BRCA1* results in loss of DNA damage–inducible FANCD2 foci, suggesting that BRCA1 may act as an "organizer" of FA foci (*8*). Accordingly, the BRCA1 protein has a Ring Finger E3 ubiquitin ligase domain and may ubiquitinate FANCD2 in vivo (*10*). Second, $BRCA1^{-/-}$ or $BRCA2^{-/-}$ tumor cells exhibit MMC hypersensitivity and chromosome instability (*11–13*), similar to the defects observed in FA cells (fig. S1). Functional complementation of $BRCA2^{-/-}$ cells with murine wild-type *Brca2* restores MMC resistance (*14*). Third, targeted inactivation of the murine *Brca2* gene, disrupting the COOH-terminus of the BRCA2 protein but sparing the NH_2-terminus, results in viable mice with an FA-like phenotype (i.e., small size, skeletal defects, hypogonadism, cancer susceptibility, chromosome instability, and MMC hypersensitivity) (*15*, *16*).

To investigate the relation between *BRCA* genes and FA, we sequenced *BRCA1* and *BRCA2* in cells derived from FA-B and FA-D1 patients (Table 1 and Fig. 1A). Although no *BRCA1* mutations were detected, biallelic mutations in *BRCA2* were observed. A homozygous mutation (IVS19-1 G to A) was detected in a *BRCA2* splice acceptor site in the FA-D1 reference line, HSC62, predicted to result in partial or complete loss of exon 20. In another FA-D1 line, EUFA423, two definitive *BRCA2* mutations were identified, 7691insAT (exon 15) and 9900insA (exon 27). These mutant alleles were not detected in a screen of 120 random genomic DNA samples from the general population. Both mutations create frameshifts and are predicted to encode COOH-terminal truncated BRCA2 proteins. The 9900insA mutant allele has previously been identified in a breast cancer kindred (Table 1) (*17*).

Paradoxically, the FA-B reference line, HSC230, also contained two abnormal *BRCA2* alleles. One mutant allele contained a known 3033delAAAC frameshift mutation in exon 11, and the second allele contained the polymorphic stop codon (ter3326) in exon 27 (*18*). This latter allele has been detected in approximately 1% of normal controls in the U.S. population and is not associated with a strong cancer risk (*18*). That FA-D1 and FA-B cells had biallelic mutations in the same gene (*BRCA2*) suggests the possibility of intra-allelic complementation or phenotypic reversion to wild-type (*6*). Two additional cell lines from FA patients of unassigned subtype had biallelic mutations in *BRCA2* (Table 1).

We next examined BRCA2 protein expression in the FA-D1 and FA-B cell lines (Fig. 1, B to D). An antibody to the COOH-terminus of BRCA2 (Ab-2) recognized full-length BRCA2 (380 kD) in normal control lymphoblasts, HeLa cells, and HSC62 (FA-D1) (Fig. 1B, lanes 1, 2, and 5). EUFA423 cells expressed a truncated BRCA2 protein (230 kD, lane 4), and no BRCA2 was detected in CAPAN1 (*19*) or HSC230 (FA-B) cells with this antibody (lanes 3 and 6). Probing again but with a different antibody (Ab-1, see epitope in Fig. 1A) revealed expression of BRCA2 in EUFA423 and HSC230 (Fig. 1C, lanes 4 and 6), suggesting that these BRCA2

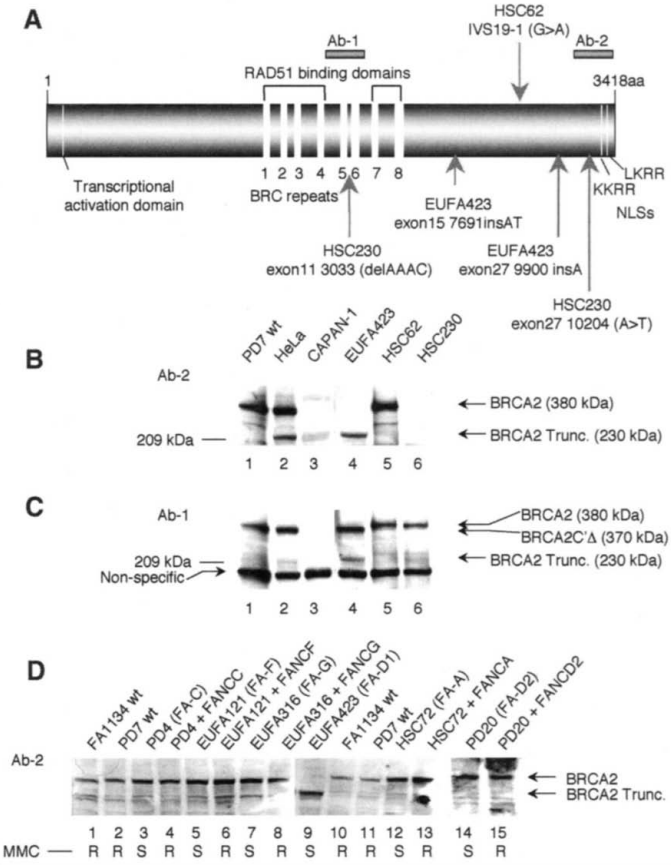

Fig. 1. FA-B and FA-D1 cells have biallelic BRCA2 mutations and express mutant BRCA2 proteins. (**A**) Schematic diagram of human BRCA2. The highly conserved BRC repeats, encoded by exon 11, mediate RAD51 interactions. *BRCA2* mutations in EUFA423 (FA-D1), HSC62 (FA-D1), and HSC230 (FA-B) lymphoblasts are shown. Mutations were confirmed in primary cells. (**B**) Whole cell lysates were prepared from wild-type PD7 lymphoblasts, HeLa cells, CAPAN1, EUFA423 (FA-D1), HSC62 (FA-D1), and HSC230 (FA-B) lymphoblasts. CAPAN1 is a pancreatic carcinoma cell line that has lost one *BRCA2* allele and contains the 6174delT mutation in the remaining allele (*19*). Proteins were separated by electrophoresis and immunoblotted with a rabbit polyclonal antibody to BRCA2 (raised against amino acids 3245–3418 of BRCA2) (Ab-2, Oncogene Research) or (**C**) by a mouse monoclonal antibody to BRCA2 (raised against amino acids 1651–1821) (Ab-1, Oncogene Research). A protein in HeLa cell extracts (209 kD) was nonspecific. (**D**) Characterization of the BRCA2 protein in FA lymphoblasts from multiple complementation groups (subtypes A, C, D1, D2, E, F, and G). Proteins were immunoblotted with Ab-2. S, MMC sensitive; R, MMC resistant.

Table 1. FA patients with biallelic mutations in *BRCA2*. U/A, unassigned FA subtype. BIC, Breast Cancer Information Core. See www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic. Dashes indicate no recorded BIC entry.

Cell line	FA subtype	Allele 1		Allele 2	
		Mutation (exon)	BIC entry	Mutation (exon)	BIC entry
HSC62	D1	IVS19-1 G to A (20)	–	IVS19-1 G to A* (20)	–
EUFA423	D1	7691/insAT (15)	–	9900/insA (27)	4
HSC230	B	3033/del AAAC (11)	Many	10204 A to T† (27)	Many
EUFA579	U/A	7235 G to A (13)	1	5837 TC to AG (11)	1
AP37P	U/A	8415 G to T (18)	2	8732 C to A (20)	1

*Family history of consanguinity. †Polymorphic STOP variant (ter3326).

polypeptides are truncated at the COOH-terminus (BRCA2C'Δ, 370 kD). Ab-1 also recognized the truncated BRCA2 protein (BRCA2-Trunc) in EUFA423 (lane 4), suggesting that this isoform has an internal deletion between the two antibody epitopes. Taken together, these results indicate that EUFA423 and HSC230 express BRCA2 polypeptides with small COOH-terminal truncations, consistent with the presence of mutations in exon 27 (Table 1). Cell lines from other FA subtypes displayed approximately equal levels of full-length BRCA2 (Fig. 1D).

Although HSC62 cells express BRCA2 protein of approximately normal size, the mutation (IVS19-1 G to A) predicts the presence of an abnormally spliced messenger RNA (mRNA). To test this, we performed reverse transcriptase–polymerase chain reaction (RT-PCR) of the *BRCA2* mRNA, followed by direct cDNA sequencing (Fig. 2, A to C). As a result of this mutation, the *BRCA2* mRNA lacks the first 12 bases of exon 20, corresponding to an in-frame deletion of four amino acids from BRCA2 (amino acids 2830 to 2833) (Fig. 2C, fig. S2). No normal *BRCA2* mRNA was detected in HSC62 cells. The mutant protein may have partial activity because the HSC62 patient has a relatively mild clinical FA phenotype (table S1) and the HSC62 cells have only modest MMC sensitivity (*20*) (Table 2).

We next determined, by means of genomic PCR with specific flanking primers and direct sequencing, whether the *BRCA2* mutant alleles segregate in the EUFA423 kindred (Fig. 3). The paternal allele was 7691insAT and the maternal allele was 9900insA (Fig. 3A). The proband (EUFA423) was a compound heterozygote, whereas two of the three unaffected siblings were *BRCA2* carriers. Lymphoblasts from all heterozygous *BRCA2* carriers expressed full-length BRCA2 (Fig. 3B).

Next, we stably transfected EUFA423 fibroblasts with cDNA encoding the full-length wild-type BRCA2 protein (Fig. 3C). G418-selected cells expressed full-length BRCA2 (Fig. 3C, lane 3) and exhibited a correction of their MMC sensitivity (Table 2). Similarly, transfection with human chromosome 13, which contains the wild-type *BRCA2* gene, corrected the MMC hypersensitivity (Table 2). Taken together, these results confirm that *BRCA2* is a FA gene.

FA has an estimated incidence of less than 1 per 100,000 live births, and less than 5% of FA families are assigned to subtypes B and D1. *BRCA2* mutations have a cumulative carrier frequency of approximately 1% of the U.S. population (*17*). This *BRCA2* carrier frequency predicts a higher incidence of *BRCA2* homozygotes than the observed FA incidence. On the basis of our limited sample collection, FA patients have at least one mutation in the 3′ region of *BRCA2*. Thus, only a subset of $BRCA2^{-/-}$ individuals (namely, those expressing truncated BRCA2 proteins with partial activity) may manifest the FA phenotype. Homozygous disruption of the 5′ end of *BRCA2*, in contrast, may result in embryonic lethality, as it did in the mouse model (*21–23*).

Specific *BRCA2* mutations may vary in cancer risk (*17*). The 6174delT mutation found in Ashkenazi Jews may confer a breast

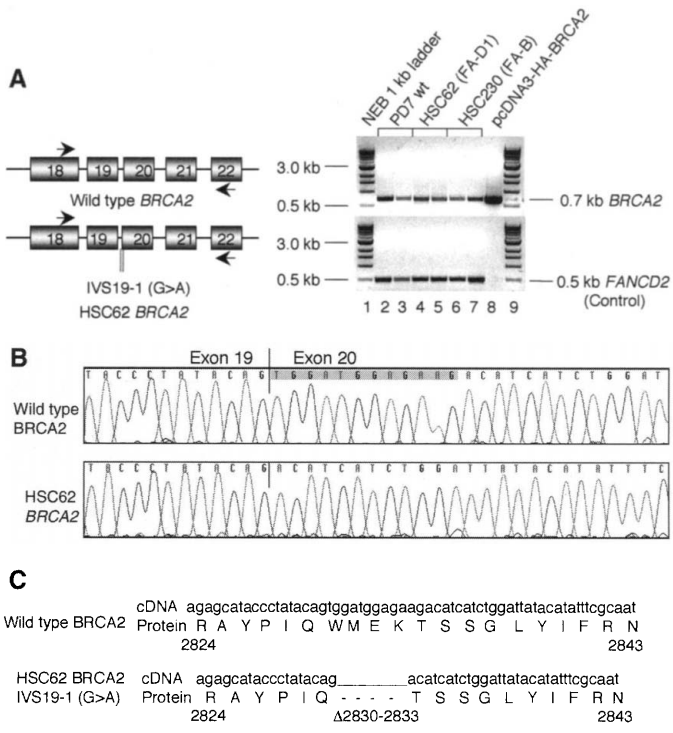

Fig. 2. The FA-D1 reference line, HSC62, expresses a BRCA2 protein with an internal deletion of four amino acids. (**A**) Schematic representation of the RT-PCR reaction, resulting in specific amplification of a region of the *BRCA2* mRNA. PCR products from the indicated cell lines were analyzed on a 1% agarose gel. (**B**) PCR products were analyzed by direct DNA sequencing. (**C**) *BRCA2* mRNA from HSC62 cells has an internal deletion of the first 12 bases from exon 20, resulting in an in-frame deletion of the indicated four amino acids. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; W, Trp; and Y, Tyr.

Table 2. Chromosome breakage analysis of FA and control cell lines was performed as in (*7*). Groups of experiments for lymphoblasts are separated by a line space. I and II indicate experiment number for fibroblast cell lines. S, MMC-sensitive; R, MMC-resistant; L, EBV-transformed lymphoblasts; F, SV-40 transformed fibroblasts; ND, not determined; wt, wild type.

Cell line	MMC (ng/ml)	% Cells with radials		Phenotype
		I	II	
	Lymphoblasts			
PD7L (wt)	20	8		R
EUFA121L (FA-F)	20	45		S
HSC62L (FA-D1)	20	38		S
EUFA423L (FA-D1)	20	91		S
HSC230L (FA-B)	20	94		S
PD7L (wt)	20	2		R
EUFA121L (FA-F)	20	64		S
EUFA423L (FA-D1)	20	90		S
EUFA424L	20	8		R
EUFA425L	20	0		R
EUFA664L	20	4		R
EUFA665L	20	6		R
EUFA666L	20	ND		
	Fibroblasts			
GM0637F (wt)	25	10	6	R
GM6914F (FA-A)	25	64	49	S
EUFA423F (FA-D1) + pcDNA3-empty	25	88	91	S
EUFA423F (FA-D1) + pcDNA3-HA-BRCA2	25	24	28	R
EUFA423F + human chromosome 13	25	9	15	R

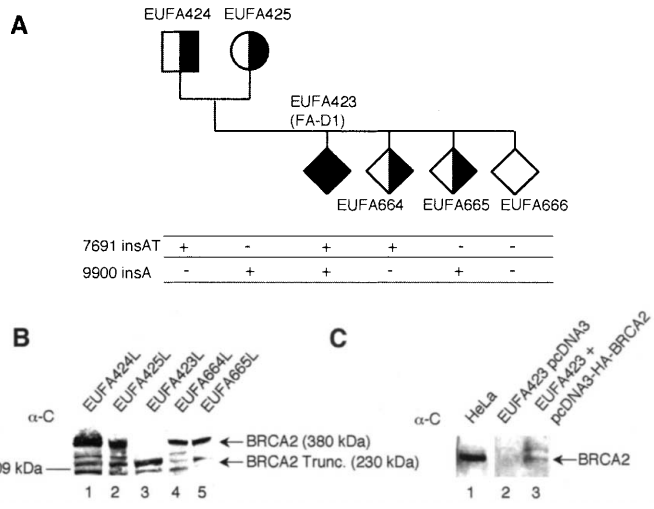

Fig. 3. Segregation of *BRCA2* mutant alleles in the EUFA423 pedigree. **(A)** The proband with FA subtype D1 is EUFA423. Genomic DNA was prepared from lymphoblasts from the indicated family members and was sequenced for *BRCA2* mutations. **(B)** Expression of mutant BRCA2 polypeptides in lymphoblasts derived from EUFA423 kindred. Proteins were immunoblotted with Ab-2. **(C)** EUFA423F was transfected either with pcDNA3-empty vector or pcDNA3-HA-BRCA2 (*28*, *29*), and stable G418-resistant cells were isolated. Cell lines were analyzed by immunoblot with Ab-2 and by the MMC chromosome breakage assay (Table 2).

cancer risk as high as 70% by age 70. Other variant *BRCA2* alleles, such as the polymorphic stop codon ter3326, appear to cause no increased cancer risk (*18*) but may cause FA in the compound heterozygous state. The smallest known cancer-associated deletion removes only 224 amino acids from the COOH-terminus of BRCA2 (*24*). Due to the unavailability of clinical records, we were unable to assess the cancer risk of the *BRCA2* mutant alleles in these FA families (Table 1).

FA patients with biallelic *BRCA2* mutations share clinical features with FA patients from other subtypes (i.e., congenital abnormalities, abnormal skin pigmentation, bone marrow failure, and cellular sensitivity to MMC) (*25*) (table S1). These similarities suggest that BRCA2 and other FA proteins cooperate in a common DNA damage response pathway, the FA/BRCA pathway (Model, fig. S3A). According to this model, DNA damage activates the monoubiquitination of FANCD2, thus targeting FANCD2 to DNA repair foci containing BRCA1 and BRCA2 (*26*). Previous studies have indicated that FA-B cells lack FANCD2 monoubiquitination, whereas FA-D1 cells express monoubiquitinated FANCD2 (*8*) (fig. S3B). BRCA2 may function upstream in the pathway, by promoting FA complex assembly and FANCD2 activation, and/or downstream in the pathway, by transducing signals from FA proteins to RAD51 and the homologous recombination machinery (*27*). The precise molecular function(s) of BRCA1 and BRCA2 in this pathway remain to be elucidated.

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Table S1

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Activation of Central Melanocortin Pathways by Fenfluramine

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D-fenfluramine (d-FEN) was once widely prescribed and was among the most effective weight loss drugs, but was withdrawn from clinical use because of reports of cardiac complications in a subset of patients. Discerning the neurobiology underlying the anorexic action of d-FEN may facilitate the development of new drugs to prevent and treat obesity. Through a combination of functional neuroanatomy, feeding, and electrophysiology studies in rodents, we show that d-FEN–induced anorexia requires activation of central nervous system melanocortin pathways. These results provide a mechanistic explanation of d-FEN's anorexic actions and indicate that drugs targeting these downstream melanocortin pathways may prove to be effective and more selective antiobesity treatments.

Drugs that increase the activity of central serotonin (5-hydroxytryptamine, 5-HT) have been widely used as appetite suppressants (*1*–*3*). However, these drugs often elicit unwanted side effects because they target multiple 5-HT pathways and receptors. A notable example is d-FEN, a drug that blocks the reuptake of 5-HT and stimulates its release (*3*). In the mid-1990s, d-FEN was prescribed to millions of people in the United States for weight loss, frequently in combination with the sympathomimetic phentermine, but was