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Chromosome fragility: molecular mechanisms and cellular consequences

Catherine H. Freudenreich

Department of Biology and Program in Genetics, Tufts University, Medford MA 02155

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1. ABSTRACT

Fragile Sites are regions of genomes that are prone to breakage. In human cells, rare fragile sites are due to expansion of repetitive sequences which have been either shown or predicted to form DNA secondary structures such as hairpins, cruciforms, and quadruplexes. For human common fragile sites, which are components of normal chromatin structure, are induced by replication inhibitors, and encompass much larger regions (100s-1000s of kilobases) it has been more difficult to define particular sequence elements responsible for fragility. However recent progress reviewed here in understanding the link between replication and fragility, as well as identification of proteins and conditions needed to prevent chromosome fragility, have shed some light onto the reasons for breakage at common fragile sites. In addition, the discovery of several types of natural fragile sites on yeast chromosomes and the characterization of associated deletions, duplications, and translocations, has revealed potential mechanisms for fragility and for the chromosomal rearrangements that follow. An understanding of these events will provide insight into the generation of cancer, since deletions and rearrangements at human common fragile sites and associated tumor suppressor genes are an early event in tumorigenesis.

2. INTRODUCTION

Chromosomes can be under a lot of stress. They are bombarded by exogenous chemicals and endogenous nucleases. Histones are moved and DNA strands separated for replication and transcription. Then they have to condense enormously and be pulled to the edges of the cell during mitosis. Recent progress has shed light on our understanding of how and why chromosomes break in the first place and the molecular mechanisms involved. This review will focus on the mechanisms of chromosome fragility that operate at regions of the genome known to be particularly prone to breakage, known as "fragile sites", and the cellular consequences of chromosome breakage at these regions.

3. CAUSES OF CHROMOSOME BREAKAGE

There are several known causes of chromosome breakage that may be applicable to fragile sites. One cause of chromosome breakage that is thought to occur as a normal part of each cell cycle is breakdown of stalled replication forks. It was first shown in bacteria that stalled forks can lead to double-strand breaks (DSBs) (73), and similar events appear to occur in eukaryotes (13, 81). There are two models for how a stalled fork can be

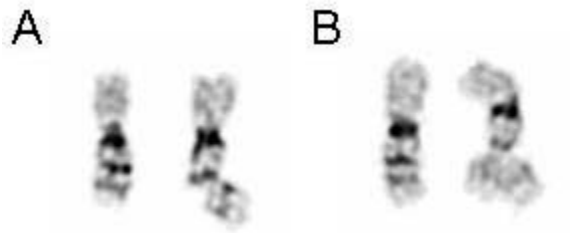

Figure 1. Cytogenetic expression of FRA16B rare fragile site. (A) Normal chromosome 16 (left), and FRA16B expressing chromosome (right). (B) Normal chromosome 16 (left), and a duplication of chromosome 16 distal to FRA16B (right) from a different cell of the same individual as in (A). The duplication most likely arose after breakage of the fragile site, followed by nondisjunction of the acentric fragment during mitotic anaphase. Picture kindly provided by Janet Cowan, Tufts University School of Medicine.

converted to a DSB. First, the single-strand DNA and the branched DNA structure present at a stalled fork are inherently less stable than double-stranded DNA, and are likely more prone to mechanical breakage or action by nucleases. Secondly, active processing of Holliday junction-like structures that can form from reversed forks by a nuclease such as RuvAB can lead to a DSB (96).

A second mechanism for generation of breaks is the conversion of a single-strand break (SSB) to a double-strand break (DSB). This can occur by nuclease processing of a SSB lesion, for example Vispe et al. (2003) have used an in vitro system to show that two closely spaced single-strand interruptions produced by base excision repair enzymes can be converted to a DSB by the action of flap endonuclease 1 (FEN-1) (108). Replication of a nicked chromosome is another mechanism where a SSB can be converted to a DSB (25).

A third mechanism of chromosome breakage can occur during anaphase of mitosis or meiosis II, when chromatids separate and are pulled to opposite poles. Dicentric chromosomes pulled to two opposite poles will break, as will chromatids which are not completely replicated. Recent experiments using live cell analysis showed that for chromatids that have undergone end fusion to create a dicentric chromosome, the resulting anaphase bridges are severed in the middle between the two centromeres (97). This is interesting because the point of breakage maps to the site of the predicted cruciform DNA structure, rather than being randomly located as predicted if mechanical force alone were responsible. (97).

Chromosome breakage is a major threat to genome integrity, and so the cell has devised multiple redundant pathways to prevent chromosome breaks from happening and heal breaks once they occur. Pathways to prevent breakage include replication fork pausing when a lesion or other difficult-to-replicate structure is encountered followed by fork restart, or repair of SSBs or gaps by any number of pathways to prevent conversion of the SSB to a DSB. Once a DSB is formed, the cell may heal the break with minimal or no changes to the DNA sequence by either non-homologous end-joining or homologous recombination. Alternative methods of healing such as deletion by single-strand annealing, translocation, or addition of a new telomere to cap the broken end lead to loss of sequences and undesirable chromosomal rearrangements. It is these undesirable healings that can lead to the cellular dysfunction of cancer cells and promote tumorigenesis. Indeed, most tumor cells exhibit multiple chromosome deletions and rearrangements.

4. COMMON AND RARE FRAGILE SITES ARE BREAKAGE PRONE REGIONS

The mechanisms outlined above, fork stalling, SSB formation, and breakage of chromatids under tension, may not be sequence independent. Indeed, there are many documented regions in the human chromosome that appear to be more prone to breakage and subsequent rearrangement than the average sequence. Human "fragile sites" are defined as regions that appear as gaps or breaks on metaphase chromosomes at a higher than average frequency, and they are exacerbated by conditions of replicative stress (4, 37, 89, 95). The cytogenetically visible fragile sites may not always be actually broken, but could represent uncondensed regions of the chromosome, for example due to unreplicated or single-stranded DNA. However, there is also clear evidence that these "gaps" can and often do lead to a broken chromatid (see below and Figure 1). Traditionally, fragile sites have been divided into two categories, "rare" and "common".

4.1. Rare fragile sites

Rare fragile sites are present in less than 5% of the population and are caused by expansion of a repetitive DNA element (see (103) for review). For example, expansion of a CGG·CCG repeat is the cause of the folate-sensitive fragile sites FRAXA, FRAXE, FRAXF, FRA16A and FRA11B. Apart from their fragility, the CGG·CCG repeat expansions at both FRAXA and FRAXE cause X-linked mental retardation by interfering with expression of a downstream gene. Breakage at the FRA11B sequence has been shown to give rise to some cases of Jacobsen syndrome, providing evidence that expanded CGG·CCG sequences also break *in vivo* in humans (57). Additional evidence for *in vivo* breakage comes from yeast, since an expanded CGG·CCG repeat integrated into a yeast chromosome causes length-dependent chromosome fragility (7). Like CGG·CCG repeats, CAG·CTG repeat expansion can also cause disease in humans, and expanded CAG·CTG repeats act as length-dependent fragile sites when inserted into a yeast chromosome both in mitotic cells (12, 35) and during meiosis (55, 56). Although fragile sites have not been detected on metaphase spreads at expanded CAG·CTG sequences in human cells (54, 114), an increased number of micronuclei have been detected in lymphocytes from myotonic dystrophy patients with CAG·CTG expansions, suggesting chromosomal instability at the expanded repeat sequence (14). A comparison of breakage rates in yeast at CGG·CCG and CAG·CTG repeats of comparable lengths indicates that CGG·CCG sequences have at least a 10-fold higher rate of breakage,

suggesting that breakage at CAG·CTG repeats may also occur in human cells but at a level difficult to detect by cytogenetics ((7); B.A. Lenzmeier and V.A. Zakian, personal communication).

Two other rare fragile sites, FRA10B and FRA16B, are caused by expansion of AT-rich minisatellite repeats that are highly related (~42 bp and ~33 bp respectively) (48, 115) (Figure 1). Expression is inducible by the nucleotide analog bromodeoxyuridine for FRA10B and by the minor groove binder distamycin A for FRA16B. A common feature of the sequences that form rare fragile sites is that they are able to form secondary structures. CTG, CAG and CCG repeats can form stable hairpin structures, and CGG repeats can form hairpins or quadruplexes (Figure 2A, B) (see (71) for review). A consensus sequence for both the FRA10B and FRA16B AT minisatellite repeats contains a 19 bp inverted repeat (Figure 2C) (48), and a structure predicted by the Mfold program using a normal (unexpanded) FRA10B allele predicts a complex secondary structure with multiple hairpins (Figure 2D).

4.2. Common fragile sites

Common fragile sites are found in all individuals, and thus represent a component of normal chromatin structure (see (37, 89) for review). There are about 80 common fragile sites that occur at different frequencies, with the 20 most fragile sites accounting for the majority (80%) of gaps and breaks (4). Most common fragile sites are inducible by aphidocolin, an inhibitor of polymerases α, δ and ε. In vivo, their expression may be induced by environmental or dietary factors, as their expression can be enhanced by caffeine, ethanol, and hypoxia (24, 89). In addition, deletions at or near fragile site regions have been documented in many cancer cell lines (51). The two most highly expressed sites, FRA3B and FRA16D are located within a tumor suppressor gene, FHIT at FRA3B, and WWOX at FRA16D, and have been associated with deletions and translocations in a wide variety of tumor types, including those in tissues exposed to the environment such as the gastrointestinal tract and the lungs (51, 89).

Thirteen human common fragile sites have been characterized at the molecular level (4, 95, 119). The regions of fragile site induction are generally large, spanning hundreds to thousands of kilobases. They do not appear to contain expanded repeat elements analogous to rare fragile sites. Using a computer program (FlexStab or TwistFlex) to predict the flexibility of the DNA helix, Kerem's group has shown that fragile site regions show clusters of high flexibility peaks that correlate with highly AT-rich regions (78%, versus 61% for nonflexible flanking sequences) (119). In addition, the AT-rich flexible regions contain interrupted runs of AT/TA dinucleotides that are similar to the AT-rich repeats of the FRA16B and FRA10B rare fragile sites. Indeed, the genomic regions containing the unexpanded alleles of these two rare fragile sites can be induced as common fragile sites by aphidocolin (119). Thus, the molecular basis of rare and common fragile sites may actually be the same or very similar. One good candidate for that common molecular element is secondary-structure forming sequences (Figure 2; Fig 3). Analysis of the 294 bp AT-dinucleotide flexibility island of FRA7E by the Mfold program shows folding into a secondary structure that contains multiple hairpin loops (119). A similar analysis shows that the peak of highest flexibility within FRA16D, which contains an AT repeat embedded in an AT-rich region, can also form a stable secondary structure (Figure 2E).

4.3. Other types of fragile regions

Although not classified as rare or common fragile sites by cytogenetic criteria, a number of other sequences have been identified that are breakage-prone regions because they cause chromosome rearrangements *in vivo* or cause chromosome breakage as assessed by other types of assays. For example, one of the most frequently occurring translocations in the human germline is between specific loci on chromosome 11 and 22. Sequences on both chromosomes at the translocation site contain palindromic AT-rich repeats (~90% A/T) that can form a large cruciform structure (the most common allele on 11 is 445 bp) (65, 66). The translocation breakpoint maps to the tip of the cruciform structure, suggesting a structure-specific nuclease cleavage may initiate the breakage and translocation event (65). The likelihood of translocation is proportional to the length and symmetry of the palindrome, showing that genetic variation strongly influences the likelihood of the t(11:22) translocation (58). Site-specific chromosome breakage also occurs at closely spaced human Alu sequences inserted on a yeast chromosome in a head-to-head orientation so that there is the potential to form a hairpin or cruciform structure (72). In this case, endonuclease cleavage was found to be at the base of the hairpin and processing of the broken end was dependent on the Mre11 nuclease (72).

In addition to sequences predicted to form secondary structures, other types of non-B DNA sequences have also been associated with chromosome breakage. The most common chromosomal abnormality in human cancer is a translocation between the immunoglobulin heavy-chain locus on chromosome 14 and the major breakpoint region (Mbr) at the Bcl-2 gene on chromosome 18 (87). Chemical probing with bisulphate has determined that the Mbr region has single-stranded regions that map at the nucleotide level to the same regions as the most common breakpoints (87). Nicking at the Mbr was dependent on the RAG complex, suggesting that RAG nuclease activity is able to cleave the unusual DNA structure to directly cause a DNA strand break. The single-stranded region is due to formation of a three-stranded *R*.R.Y triplex structure called H-DNA, where the fourth strand is left unpaired (86) (Figure 3). Triplex formation is essential for Mbr breakage *in vivo*, as breakage was abolished when mutations that abolish the triplex structure were introduced (86). Other naturally occurring H-DNA and Z-DNA (left-handed DNA) sequences have also been shown to induce breaks and subsequent deletions in mammalian cells (6, 109, 110). These breaks likely have functional consequences as well, for example translocation and deletion breakpoints map to the H-DNA and Z-DNA forming sequences at the c-myc oncogene promoter.

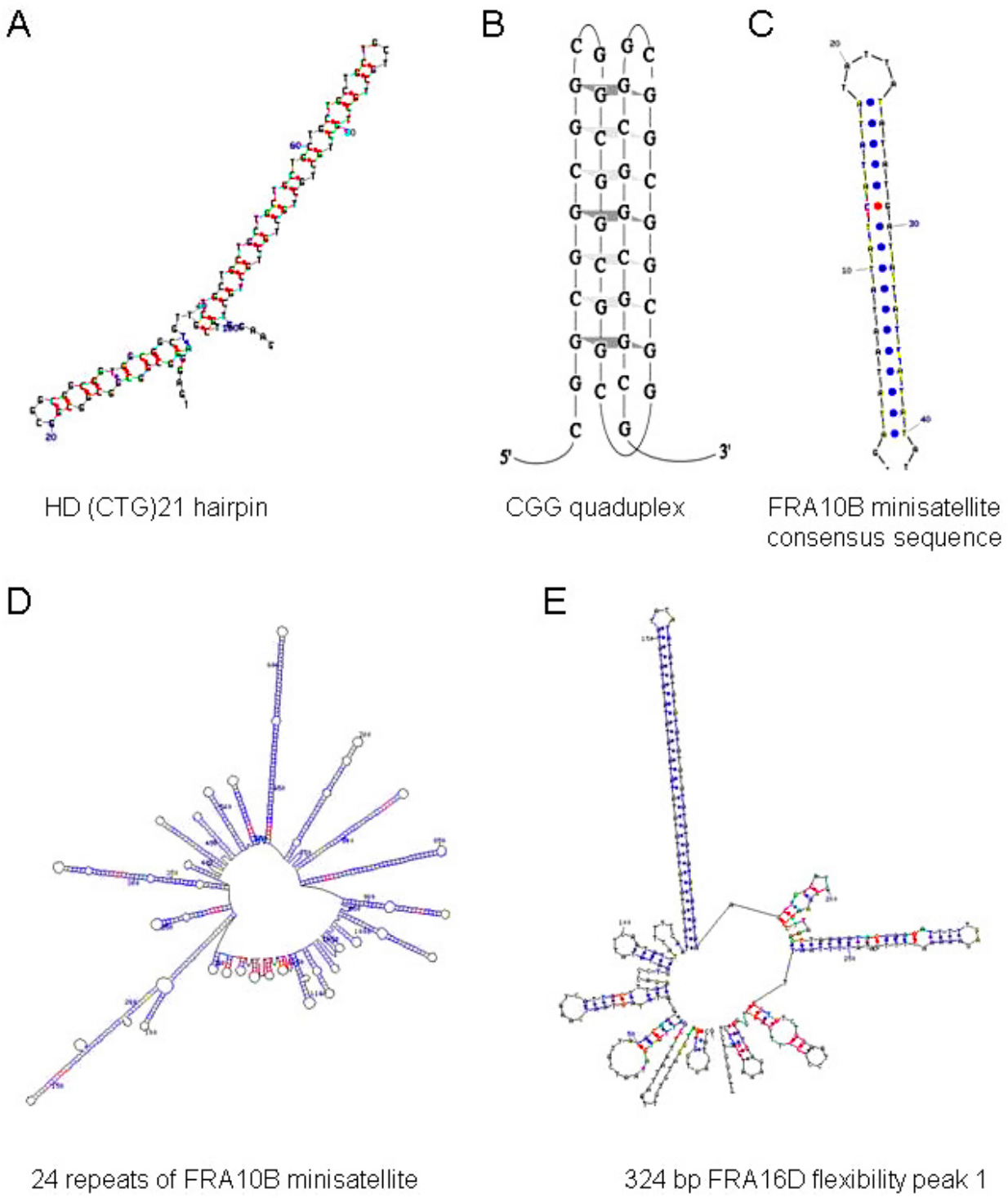

Figure 2. Predicted secondary structure formation for fragile site sequences. A,C,D,F are predicted by Mfold http://www.bioinfo.rpi.edu/applications/mfold/dna/ (120). In each case, the highest Tm structure is shown. (A) 21 CTG repeats from a normal allele of the Huntingtin (HD) gene obtained from GenBank; the predicted Tm by Mfold is 84.5 °C. (B) Quadruplex formed by CGG repeats based on a projected structure of two associated CGG hairpins (60) kindly supplied by Karen Usdin of LMCB, NIH. (C) FRA10B 42 bp consensus repeat sequence (48). (D) Normal allele of FRA10B containing 24 repeats of the AT rich minisatellite, obtained from GenBank. The Predicted Tm by Mfold is 63.3 °C. (E) A 324 bp AT-rich sequence containing flexibility peak 1 from the FRA16D region obtained from GenBank (90). The predicted Tm by Mfold is 60 °C.

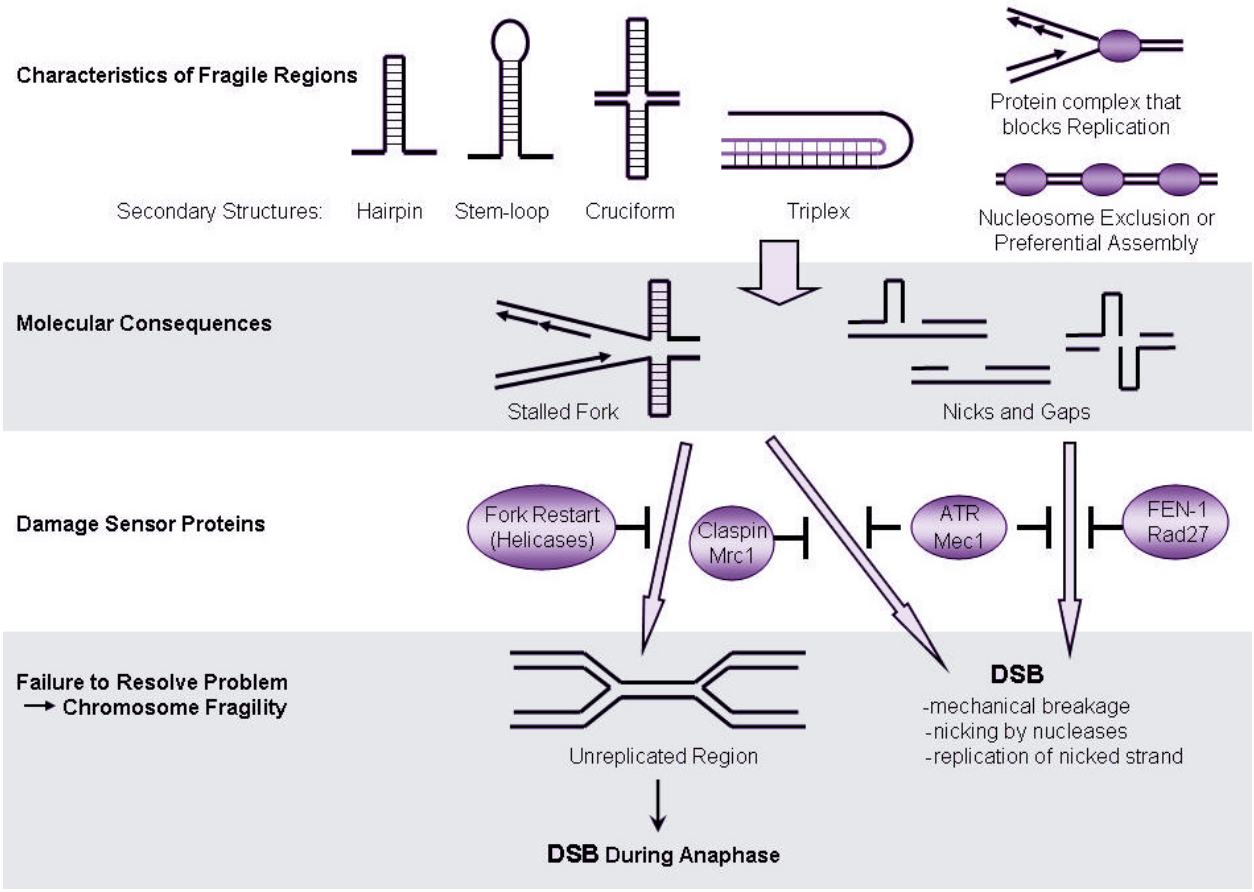

Figure 3. Proposed mechanisms of chromosome breakage at fragile sites. Characteristics of Fragile Regions: evidence summarized in this review indicates that secondary structure formation such as cruciforms, or multiple hairpins or stem-loops is common to many fragile site regions. Other characteristics found are triplex DNA (H-DNA), protein barriers to replication, and nucleosome exclusion or preferential assembly. For triplex DNA, pyrimidine-rich strands and Watson-Crick basepairs are black, the purine-rich strands and Hoogsteen basepairs are grey. Molecular consequences of the above characteristics could be stalled replication forks and nicked or gapped DNA (with or without associated secondary structures). Damage sensor proteins normally sense stalled forks or nicked DNA and reverse the damage to prevent unreplicated regions or DSBs. For example, helicases and other proteins mediate fork restart, Claspin/ Mrc1 prevents dissociation of the replisome, and ATR/ Mec1 prevents fork breakdown. FEN1/ Rad27 flap endonuclease and ssDNA sensing by ATR/ Mec1 could contribute to repair of nicks and gaps. Unreplicated DNA that persists and escapes the replication checkpoint is predicted to give rise to DSBs during anaphase. Failure of any of these mechanisms can lead directly to double-stranded breaks, some of which may repair improperly leading to deletions, duplications, or translocations.

Further insight into the types of sequences that can cause chromosome fragility has been obtained by finding natural fragile sites in budding yeast. As in humans, these sites have been identified in cells with defects in DNA replication or checkpoint controls (see more on links between fragility, replication, and checkpoint controls in the next two sections). Cha and Kleckner (17) identified several fragile sites on yeast chromosome III in a strain mutated for the yeast ATR homolog, Mec1, that they mapped to replication slow zones (RSZs). These zones slow replication fork progression as shown by two-diminensional (2D) gel analysis and appear analogous to fragile sites on human chromosomes. The RSZs do not map to origins, but may be at or near replication termination regions, and some contain clusters of tRNA genes. tRNA genes are associated with replication stalling and subsequent chromosome breakage due to collision with bound RNA polymerase III transcription complexes (53). By using a strain expressing a low level of DNA polymerase α, Lemoine *et al* (70) identified two head-to-head Ty elements (yeast retrotransposons) that were a preferred site for DSBs. These elements are very large inverted repeats (6kb, separated by 283bp), which could potentially form a hairpin or cruciform structure that could be cleaved by an endonuclease to produce a break. Presumably a very large single-stranded region would have to be exposed to allow structure formation because of the space between the inverted repeats, a condition likely made possible by inefficient replication due to low polymerase levels. Another yeast fragile site is associated with two tRNA genes known to stall replication forks as well as five long terminal repeats (LTRs; found at the ends of Ty

retrotransposons), two of which are in a head-to-head orientation (1).

The sum of these and other studies indicate that there are several types of DNA sequences that can lead to chromosome fragility: (1) structure-forming sequences, (2) sequences that assume non-B DNA structures, (3) sequences that block DNA replication via non-histone protein binding (Figure 3). The mechanisms by which these sequences can cause breakage include stalling of DNA replication followed by fork breakdown, breakage of unreplicated regions at anaphase, site-specific nuclease cleavage, or conversion of a single-strand nick to a DSB (Figure 3). It remains to be seen which of these types of sequences and mechanisms are most responsible for breakage at human common fragile sites. The evidence obtained so far points to structure-forming sequences that stall DNA replication (see below). However, much remains to be learned, and any or every combination of these mechanisms is possible.

5. THE LINK BETWEEN FRAGILE SITES AND REPLICATION

5.1. Fragile site regions appear to stall or slow replication

The two conditions that induce expression of the majority of human fragile sites, folate deprivation and aphidocolin, both inhibit DNA replication, leading to the idea that either stalled or collapsed replication forks or unreplicated DNA that persists into metaphase is the cause of fragile site expression. Supporting the idea that fork stalling is important, 2D gel analysis of expanded CGG·CCG and CAG·CTG repeat sequences analyzed in both *E. coli* and yeast cells directly demonstrated stalled or slowed replication forks that mapped to the repeat sequence (83, 92). Recent results from our lab show that a short sequence that includes the peak of highest flexibility within FRA16D (Flex1, Figure 2E) stalls a replication fork when replicated on a plasmid in yeast cells (H. Zhang and C.H. Freudenreich, unpublished data). The AT repeat embedded within the Flex1 AT-rich sequence is predicted to easily extrude to form either a hairpin (from a single-strand of DNA) or cruciform (from dsDNA) structure. The strength of the fork stalling was dependent on the length of the perfect AT repeat embedded within this AT-rich sequence, which is polymorphic in the human population (H. Zhang and C.H. Freudenreich; (33)). In addition, using a genetic assay, we showed that this same Flex1 sequence significantly increased chromosome breakage, which was further enhanced by either presence of a replication inhibitor or absence of the DSB repair protein, Rad52 (H. Zhang and C.H. Freudenreich, unpublished data). These results directly link site-specific replication fork stalling to chromosome fragility.

There is evidence that fragile site regions may sometimes not be able to complete replication, or if so not until very late in the cell cycle. In human cells, replication timing was first evaluated at the rare FRAXA site using flow cytometry of labeled cells combined with PCR analysis (44). It was found that the normal allele of the FRAXA region replicates in late S, while replication of alleles with CGG·CCG expansions was delayed until G2/M (as defined by DNA content). Interestingly, the region of replication delay was quite large, 400 kb or more on either side of the repeat expansion, suggesting that more than a single stalled fork is involved (43, 102). How the expanded repeat could change the replication timing of such a large region is an open question. Changes in chromatin structure or origin usage (see below) are two possibilities. The AT minisatellite expansions at FRA10B and FRA16B also result in a replication delay, although the region of delay for FRA10B mapped somewhat distal to the repeat expansion itself (42). Common fragile sites FRA3B and FRA16D are also both late replicating, and for FRA3B exposure to aphidocolin delays replication further, resulting in a failure to complete replication in some cells (69, 82). Interestingly, a study of FRA3B replication timing in two different cell lines showed that one allele replicated later than the other, with or without aphidocolin treatment, and that fragility was preferentially observed on the late replicating allele (111). This result suggests a possible relationship between particular alleles and fragility. Common fragile site FRA7H has a more complicated pattern of replication with allelic asynchrony that is further enhanced by aphidocolin (45). This latter pattern is consistent with a stochastic inhibition or slowing of replication, such as would happen at a sequence with the potential to stall a fork (45). All of these results support a model that incomplete replication is important in generation of fragile sites (Figure 3). Whether that delay is due to replication fork stalling at a particular sequence or a more global delay, perhaps due to several difficult-to-replicate sequences or a combination of factors, is not yet clear.

There could also be a link between replication origins and fragile sites, as peaks of high flexibility predicted by FlexStab are over-represented at mapped mammalian replication origins (105). Furthermore, FISH analysis of aphidicolin-treated hamster cells revealed fragile site expression at two origins (105). The same group has also shown that nucleotide pool levels can modify origin usage, so that a pattern of one dominant origin can switch to a pattern of many weaker origins during conditions of replication stress (3). Thus, either a change in origin usage or more persistent origin bubbles could create areas of increased susceptibility to breakage. For example, one might imagine that if a previously inactive high flexibility sequence became an active origin in the presence of aphidocolin, it could give opportunity for formation of a secondary structure that would preclude normal replication and become a fragile site. It has recently been shown that replication from a subset of yeast origins arrests in close proximity to the origin in conditions that exacerbate fork stalling, such as hydroxyurea or checkpoint-deficient mutants *mec1-1* (yeast ATR homolog) or *rad53-1* (yeast Chk2 homolog) (88). Furthermore, they showed that these regions of fork arrest resulted in DSBs in a manner dependent on replication, linking fork stalling to chromosome fragility. However, the fork arrest may not be due to the origin *per se*, but flanking sequences, as normal fork progression is restored if one such origin is moved to another chromosomal location (88).

5.2. Consequences of fork stalling and chromosome breakage

A recent study that directly observed replication forks artificially paused at either CAG·CTG or telomeric repeat DNA by electron microscopy (EM) has provided insight into the consequence of fork stalling within a repeat tract. Unlike forks paused within unique DNA, forks paused within the repeats showed a high propensity to regress and form a four-way junction or "chicken foot" structure that could block further fork progression and lead to recombinational repair (34). The fork stalling observed at expanded repeats that cause rare fragile sites has been explained by structure formation on the template strand (74). The EM result provides an alternative (but not mutually exclusive) model. Many studies of site-specific replication fork barriers have shown that a stalled fork is subject to breakage and elevated recombination (see (62, 84) for review). Fork collapse, breakage and recombination are elevated further when the cellular checkpoint response is defective, partially due to dissociation of the stalled fork and the replisome. Consistent with the link between fork stalling and elevated recombination, we found that the FRA16D Flex1 sequence that stalled replication also created a strong recombinational hotspot, whereas alleles with shorter AT tracts that did not stall replication also did not detectibly increase recombination (H. Zhang, C.H. Freudenreich, unpublished data). It is important to note that recombinational repair of a stalled fork may not always proceed via a broken DNA intermediate, but may sometimes allow fork restart without breakage. Nonetheless, breakage is certainly one outcome, as evidenced by both direct detection of broken intermediates and outcomes that can only be explained by a DSB (such as loss of a chromosome arm).

The consequences of breakage at the fragile sites identified in yeast (see section 4.3) have been carefully studied and provide a framework for understanding the deletions, translocations, and amplifications commonly seen at fragile site regions in human cells, particularly cancer cells. For example, in fission yeast, a blocked fork initiates recombination events that lead to chromosomal rearrangements similar to those found at human fragile sites (2, 68). A general theme is that sequences near to the fragile site that contain homology elsewhere in the genome are used to repair the broken chromosome via recombination. Thus, for the fragile sites identified by Lemoine et al (70) and Admire et al (1) that occurred at or near Ty or Ty-LTR elements, respectively, recombination with homologous Ty/LTR elements on other chromosomes resulted in translocations. The events were sometimes complex, accompanied by deletions, duplications, and loss of chromosome arms. Experiments in both yeast and human cells have also shed light on the relationship between chromosome breakage and gene amplification (reviewed in (41)). The recipe for amplification contains a DSB (either induced, or a natural fragile site) next to an inverted repeat: a combination that appears to allow foldback of the inverted repeat followed by replication of the remaining chromosome arm. These mechanisms can be used to explain the deletions, translocations and gene amplifications that have been observed at human common fragile site regions, and which may play a role in cancer development or progression.

Another consequence of late replication/fork stalling at fragile regions could be an interference with chromosome condensation at metaphase. For several of the replication timing experiments at human fragile sites, addition of a replication inhibitor delayed the replication of the fragile site region until what is traditionally considered as G2 phase. Fragile sites expressed cytogenetically in M phase often appear as a non-staining gap in the chromatid that could represent uncondensed chromatin. Indeed, the Debatisse lab found that a drug that induces premature chromatin condensation, calyculin A, induces breaks at common fragile sites when added in the G2 phase (29). Interestingly, they also located many fragile sites, including FRA3B, FRA16D and FRAXB, to the interface of R and G bands (as determined by staining for both R and G bands), suggesting that they may lie at a transition between early and late replicating domains. They suggest that fragile site regions are genetically programmed pause sites that regulate replication timing (29).

5.3. Chromatin structure at fragile sites

One interesting feature that could affect the replication or condensation of fragile sites is chromatin structure (Figure 3). For example, it has been shown that CGG·CCG repeats and the FRA16B AT minisatellite exclude nucleosomes (the latter only in the presence of fragile site inducer distamycin) (50, 113). Methylation further enhances CGG·CCG nucleosome exclusion (38, 113). In contrast, CAG·CTG repeats preferentially assemble nucleosomes (39, 112). The CAG·CTG expansion at the DM locus is associated with loss of an adjacent nuclease hypersensitive site and 190 CAG·CTG repeats have the ability to silence a linked transgene in mice (93). An altered chromatin structure has been demonstrated that localizes specifically to the expanded repeat (32). Whether these altered chromatin structures affect chromosome breakage or cytogenetic expression of fragile sites remains to be investigated.

6. PROTEINS INVOLVED IN SENSING STALLED REPLICATION FORKS AND DNA DAMAGE ARE IMPORTANT FOR PREVENTING BREAKAGE AT FRAGILE SITS

6.1. Proteins that prevent fragility of common fragile sites

If fragile sites are regions that stall replication forks, then the sensor proteins involved in the S phase checkpoint would be expected to recognize and respond to the stalled fork or to DNA structures related to stalled forks such as single-stranded DNA. Studies in both yeast and mammalian cells have implicated the ATR protein (Mec1 in *Saccharomyces cerevisiae*), a key sensor of single-stranded DNA, as being important in normal fork progression and in preventing collapse of stalled forks (see (13, 81) for review). For example, chromosome fragility at the replication slow zones identified in yeast (17) only occurred in cells deficient in Mec1. Also, Glover and

colleagues have shown that human cell lines with reduced

Table 1. Proteins required to prevent expression of mammalian common fragile sites

Protein required to prevent expression of CFS[1]	Protein function[2]	Reference
ATR	ssDNA sensor, fork restart, S and G2 phase DNA damage response	15
BRCA1	G2/M checkpoint	5
FANCD2	DNA damage and replication stress response	49
SMC1 (SMC3)	Cohesin subunit, phosphorylated by ATR	75
CHK1	DNA damage response and S/G2 checkpoints, Effector kinase phosphorylated by ATR	28
RAD51	DSB repair by homologous recombination	94
DNA-PKcs	DSB repair by end joining	94
Ligase IV	DSB repair by end joining	94

[1]Expression of mammalian common fragile sites, determined cytogenetically, including FRA3B and FRA16D, [2]Most of these proteins have multiple cellular functions. As far as is currently known, the functions relevant to fragile site expression are listed.

ATR function or expressing a dominant-negative ATR allele have an increased number of metaphase breaks per cell and increased expression of FRA3B, FRA16D and FRA7H common fragile sites both in the absence and presence of aphidocolin (15). These results support the idea that common fragile sites are regions normally prone to replication fork stalling, and that ATR recognizes and prevents breakage of these stalled forks (15, 20). More recently, it has been shown that downstream targets of ATR such as BRCA1, SMC1, CHK1, and FANCD2 are also required to prevent fragile site expression (Table 1). Thus, presence of an intact S-phase checkpoint pathway is crucial for preventing breakage at common fragile sites in human cells. This pathway could act directly, to prevent breakdown of a stalled fork or promote fork restart. For example, in Xenopus egg extracts, ATR (and ATM) are needed to reload Pol ε and recruit Mre11 to allow fork restart and prevent DNA breaks (106). Alternatively, the ATR pathway could be required more indirectly to delay the cell cycle to allow for replication of a late replicating region or restart of stalled forks. Clearly, these models are not mutually exclusive, and both functions may be needed. However, results from a conditional knockout of ATR in the mouse indicate that the DNA replication checkpoint as induced by aphidocolin is still intact in ATR knockout cells (11), suggesting that a direct role in recovery of stalled forks may be more important.

Interestingly, cell lines deficient in ATM and CHK2 do not show increased chromosome breakage, suggesting that DSBs arise as a secondary event (15, 28). However, deletion of proteins involved in DSB repair, both the homologous recombination pathway (Rad51) and non-homologous end joining (DNA-PKcs and Ligase IV) increases expression of common fragile sites FRA3B and FRA16D, and both DNA-PK and γH2AX foci localize to expressed fragile site FRA3B (94) (Table 1). Thus, DSB repair proteins do appear important in preventing fragile site expression, suggesting that conversion of a stalled fork or to a DSB is not a rare event. Results from our lab also indicate that DSB repair proteins are important in preventing fragility of CAG·CTG repeats (R. Sundararajan and C.H. Freudenreich, unpublished data). DNA repair proteins such as Rad51 and BRCA1 are also involved in processing replication-blocking lesions (107), so a second function may be to aid in restarting blocked forks that have not collapsed into DSBs.

6.2. Proteins that prevent fragility of expanded CAG·CTG tracts and yeast fragile sites

Experiments in yeast from our lab show that chromosome breakage at an expanded CAG·CTG tract is increased in cells defective in the DNA damage checkpoint (36, 67). Using a sensitive genetic assay, rates of breakage were determined for a yeast artificial chromosome (YAC) that contained either no repeat, a "medium-length" CAG-85 tract, or a longer CAG-155 tract in ten different genetic backgrounds. Absence of Mec1 (ATR homolog) or it's downstream target Rad9 (which has BRCA1 homology) increased fragility of CAG·CTG repeats significantly above the general increase in chromosome breakage that was observed, suggesting that CAG·CTG repeats are particularly sensitive to the absence of these proteins, in agreement with the results for common fragile sites. In addition, cells with a checkpoint-deficient allele of either the Mrc1 protein (Claspin homolog) or the downstream effector Rad53 (Chk2 homolog) showed a dramatic CAG tract-specific increase in chromosome breakage. Since Mrc1 is known to travel with the replication fork and to maintain a stable replication complex at paused forks (59), these results directly implicate maintenance of paused forks as important for preventing breakage at sequences capable of forming secondary structures. Natural fragile sites in yeast are also dependent on the S-phase checkpoint for stability. The origin-associated fragile sites were only detected in *mec1-1* and *rad53-1* mutants (88), and instability of the yeast chromosome VII tRNA-associated fragile site increased in checkpoint-deficient mutants *rad9*Δ, *rad17*Δ, and *mec1*Δ (1).

Interestingly, the genetic results in yeast revealed a difference in the two CAG·CTG tract lengths tested, with the longer CAG-155 tract being more dependent on Mrc1 function, while the CAG-85 tract was more dependent on Mec1, Rad9, and also Rad17 (a subunit of the damage-specific PCNA complex 9-1-1) for preventing breakage. These results suggest that fork stalling is more common at the longer tract length, consistent with 2D gel analysis (83, 92). Since Mec1/ATR may not sense stalled forks per se, but rather single-stranded DNA, the shorter repeat may form a different type of damage that contains ssDNA. A good candidate for this structure is an unligated Okazaki fragment. During replication of the lagging strand, displacement synthesis forms a 5' flap that is normally cleaved by the flap endonuclease FEN-1 (yeast Rad27). It is known that CTG repeats on that 5' flap can form a hairpin that is resistant to FEN-1 cleavage, leaving an unligatable nick (47, 100). In fact, deletion of yeast FEN-1 leads to a large increase in CAG·CTG tract fragility, consistent with the predicted increase in unligated nicks (12). Formation of this structure at the CAG-85 tract is consistent with a role for Rad17, which is important for

repair of nicked or gapped DNA (30). Therefore an incompletely replicated lagging strand containing unligated nicks is another type of damage that could contribute to breakage at fragile sites (Figure 3).

Many assays designed to detect genome rearrangements have been employed to find factors important for general chromosome integrity (see (63) for review). For example, an assay designed to detect rearrangement on the unessential left arm of *S. cerevisiae* chromosome V has been used to test the effect of an extensive number of genes on gross chromosomal rearrangements, including genes involved in the DNA damage, replication, and mitotic checkpoints as well as genes involved in replication, repair, telomere addition and chromatin assembly (63, 76). Some of these gene products are needed for healing of breaks, but many are involved in maintaining chromosome integrity to prevent breakage. These types of experiments provide valuable insights into the proteins and pathways that are important in preventing general chromosome breakage, many of which will be applicable to fragile regions.

7. THE LINK BETWEEN FRAGILE SITES AND CANCER: *IN VIVO* SIGNIFICANCE

Breakage at common fragile sites has been clearly linked to cancer (see (51, 89) for review), and many cancer cell lines show a general phenotype of chromosome instability with an accumulation of translocations and deletions. Thus there is a need for a better understanding of the mechanisms behind chromosome fragility and their relation to generation and progression of cancer. In addition, many environmental factors seem to affect chromosome breakage, such as dietary factors, caffeine, ethanol, cigarette smoke, and hypoxia (24, 89, 101). There is recent evidence that the cellular pathways that monitor damage during DNA replication are key for preventing cells from starting down the road to cancer, and that inactivation of the DNA damage and checkpoint response is crucial for allowing early precancerous lesions or other cells undergoing uncontrolled division to progress to cancer (8, 40). Importantly, these precancerous proliferating cells frequently contain chromosome rearrangements at fragile sites (as compared to other regions), making these sites some of the earliest chromosomal changes associated with cancer. The implication is that replication stress at fragile sites and subsequent DSBs may be a trigger for activation of the DNA damage response, including both the ATR-γH2AX- SMC1-Chk1 and the ATM-γH2AX-Chk2-p53 pathways, and that errors in DNA repair at these sites will preferentially lead to allelic imbalances at common fragile sites because they are most sensitive to replication stress (8, 40).

Expression of fragile sites has also been shown to trigger gene amplification by breakage-fusion-bridge (BFB) cycles which can lead to amplification of oncogenes during tumor progression (23, 46). For example, breakage at FRA7I has been shown to initiate BFB cycles which leads to amplification of the PIP oncogene (21). Studies in both yeast and mammalian cells have shown that gene amplifications can occur when a break is located near an inverted repeat. In this situation, a broken single-stranded end containing the repeat can fold back to form a hairpin-capped end that can then join to the other DNA strand. Replication of a hairpin-capped chromosome arm containing a centromere will lead to a large dicentric duplication which will then show further instability via BFB cycles (41, 104). Therefore breakage caused by a fork stalled at a structure-forming repeat sequence, such as the Flex1 sequence within common fragile site FRA16D (Figure 2E), could explain the observed link between fragile sites and gene amplification.

If it weren't bad enough that fragile sites were prone to breakage and the chromosome rearrangements that can follow, several of them have been shown to be within or near tumor suppressor genes or oncogenes. FRA3B, the most highly expressed fragile site, is within a large intron of the FHIT tumor-suppressor gene (78, 99). Homozygous and hemizygous FHIT deletions are common in cancer cell lines and primary cancers, and reduced or absent Fhit expression has been reported in a wide variety of cancers (see (51, 52) for review). Loss of Fhit expression is very common in some kinds of cancer, for example reduced or absent Fhit staining was observed in 71% of invasive cervical cancers and 75% of lung tumors of smokers (22, 85). There seems to be a link between tumors caused by environmental carcinogens and alteration of the FHIT gene (52). Similarly, the second most highly expressed human fragile site, FRA16D, lies within a large intron (260 kb) of the WWOX tumor suppressor gene (9, 90). Wwox is a proapoptotic WW domain-containing oxidoreductase that binds to p53 (18, 19). Deletions in the WWOX gene and aberrant transcripts have also been observed in a number of different cancer cell lines and tumor cells, including carcinomas of the breast, ovary, colon, lung and stomach (80). In one study, 60% of invasive breast carcinomas had negative or weak Wwox expression, whereas all normal breast epithelial samples expressed Wwox abundantly (77). Ectopic WWOX expression inhibits tumor growth of breast cancer cells in a mouse model and inhibits anchorage-independent growth of breast cancer cell lines (9). In addition, the MAF oncogene is adjacent to FRA16D, and 25% of multiple myeloma cases are caused by a t(14;16) translocation that maps to MAF (64).

In addition to FRA3B and FRA16D, several other fragile sites are near to or within genes that may play a role in cancer. Another active fragile site, FRA6E, maps within a gene-rich region that includes the Parkin gene, implicated in autosomal recessive juvenile parkinsonism (16). Parkin may also be a tumor suppressor gene, as it has been found downregulated or absent in breast, ovarian, lung tumors (16, 27). Genes near FRA7G include CAV1, CAV2, (encoding caveolins 1 and 2) and TESTIN, which show loss of heterozygosity in ovarian, breast and prostate cancer with some functional evidence for a role in tumor suppression, and the oncogene MET, which is amplified in some cancers (117). The recently characterized FRA4F contains another very large gene, GRID2, which has been found deleted in hepatocellular carcinomas (10, 91).

Another interesting characteristic of common fragile sites is that they are preferred viral integration sites. An analysis of human papillomavirus (HPV) integration sites found that over half were in common fragile site regions (116). Since this virus causes cervical cancer, the presence of fragile regions in the genome may facilitate development of cervical cancer. In fact, FRA8C, which is near the c-MYC oncogene, is a target for HPV18 integrations and translocations in cervical carcinomas (31).

Breakage at rare fragile sites has not yet been associated with cancer, however, breakage at rare fragile sites followed by unfaithful repair is likely to be one mechanism of generating repeat expansions, which are the cause of several genetic diseases including fragile X syndrome, myotonic dystrophy, Huntington's disease, and multiple spinocerebellar ataxias (see (71) for review).

8. PERSPECTIVE

Although significant progress has been made, there are still many questions remaining about the causes of fragile sites and the cellular consequences. One mysterious observation is that many of the highly expressed human common fragile sites are associated with very large genes: 6 of the 10 largest genes contain a common fragile site (98). These genes span 1-2 Megabases and are mostly (>99%) intronic (98). Another question relates to the interplay of sequence characteristics and other epigenetic factors that determine whether a region will be fragile. Our demonstration of a sequence which increases fragility and causes a replication fork stall in FRA16D, together with observations of AT-rich sequences at other fragile sites, suggests that replication fork stalling at structure-forming sequences is one important piece of the puzzle. However, there are other chromosomal regions with similar sequence characteristics that don't show fragility. For example, breakage at the AT-rich palindrome at the t(11;22) translocation appears to be restricted to meiotic cells, and is not observed as a somatic aphidocolin-sensitive fragile site. Therefore, there must be other yet-undiscovered factors that influence fragility. Perhaps the association with genes is a hint since transcription has been shown to facilitate structure formation in *E. coli* (26). Chromatin structure or modifications within fragile regions is another relatively unexplored area.

Could fragile site regions also serve some important cellular function? It appears that common fragile sites may be the very last chromosomal regions to replicate (29). As such, Debatisse *et al* have proposed that their replication may act as a signal to the cell that replication is completed, and license mitotic entry (41). Also, the finding that they are some of the first sequences to be rearranged in cancer implies that they may be expressed in situations of uncontrolled cellular proliferation and serve to activate the DNA damage response to protect cells against further mutations that would lead to cancer. Smith and colleagues have proposed that the genes found at common fragile sites are part of a cellular stress response (98, 118). They found increases in the amount of RNA message produced from RORA, a large gene at FRA15A, in response to a variety of cellular stresses: aphidocolin, UV, H_2O_2, and MMS. RORA encodes an orphan retinoic acid receptor and is involved in the cellular response to hypoxia (118). Fhit hydrolyzes diadenosine tetraphosphates which are produced in cells in response to stress (61). Wwox is an oxidoreductase which also protects against ionizing radiation (79). Whether these genes and other genes at common fragile sites are somehow regulated by the status of the DNA at the fragile region or are just constitutively protective, explaining their roles as tumor suppressors, is an open question. Fragile regions may end up teaching us much about both normal cellular metabolism and pathways to cancer.

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Send correspondence to: Dr Catherine H. Freudenreich, Department of Biology and Program in Genetics, Tufts University, Medford MA 02155, Tel: 617-627-4037, Fax: 617-627-3805, E-mail: catherine.freudenreich@tufts.edu

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