Published in final edited form as:

Adv Exp Med Biol. 2017 ; 1042: 489–526. doi:10.1007/978-981-10-6955-0_21.

Fragility Extraordinaire: Unsolved Mysteries of Chromosome Fragile Sites

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Abstract

Chromosome fragile sites are a fascinating cytogenetic phenomenon now widely implicated in a slew of human diseases ranging from neurological disorders to cancer. Yet, the paths leading to these revelations were far from direct, and the number of fragile sites that have been molecularly cloned with known disease-associated genes remains modest. Moreover, as more fragile sites were being discovered, research interests in some of the earliest discovered fragile sites ebbed away, leaving a number of unsolved mysteries in chromosome biology. In this review we attempt to recount some of the early discoveries of fragile sites and highlight those phenomena that have eluded intense scrutiny but remain extremely relevant in our understanding of the mechanisms of chromosome fragility. We then survey the literature for disease association for a comprehensive list of fragile sites. We also review recent studies addressing the underlying cause of chromosome fragility while highlighting some ongoing debates. We report an observed enrichment for R-loop forming sequences in fragile site-associated genes than genomic average. Finally, we will leave the reader with some lingering questions to provoke discussion and inspire further scientific inquiries.

Keywords

Chromosome fragility; Common and rare fragile sites; DNA double-strand breaks; DNA replication stress; R-loops; Aphidicolin; Folate stress; Neurological disorders; Cancer

21.1 Introduction

Chromosome fragile sites are defined as gaps, constrictions, or breaks on metaphase chromosomes that are induced by various DNA replication inhibitors. The Human Genome Database currently documents 120 chromosome fragile sites (30 of the rare type and 90 of the common type, Tables 21.1 and 21.2). According to the HUGO Gene Nomenclature Committee, each fragile site is reported as “FRA” followed by the chromosome number and a letter denoting the order of nomenclature, such as FRA1A. In the last decade, fragile sites

have been the subject of many comprehensive reviews with respect to the molecular basis, mechanisms of expression, and relevance to human diseases (Debacker and Kooy 2007; Durkin and Glover 2007; Fungtammasan et al. 2012; Lukusa and Fryns 2008; Mirkin 2006; Thys et al. 2015). Here we approach this subject from an alternative angle. By retracing some of the early developments in fragile site discoveries, we hope to identify the gaps in our knowledge and bring awareness to some “cold cases,” so to speak, in chromosome fragile sites (we apologize to countless researchers whose work may have been omitted from this review due to space limitation). We review the classification of common vs. rare fragile sites and provide some insights into the DNA structural determinants of fragility. We also provide updated information regarding disease associations of known fragile sites in the database as well as summarize recent genome-wide studies of fragile sites. Finally, we highlight three “unsolved mysteries” in chromosome fragile sites and provide speculations.

21.2 Early History of Chromosome Fragile Site Discoveries Revisited

21.2.1 Discovery of the First Fragile Site

Early history of chromosome fragile site discoveries makes a captivating read. The term “fragile site” was coined in 1969 by Frederick Hecht to describe hitherto reported spontaneous breaks at a specific site in a human metaphase chromosome. However, the first known fragile site was observed 4 years before the genesis of its terminology (Dekaban 1965). Notably, the definition of fragile site later expanded to include constrictions and gaps in the chromosome as well. In this broader definition, the first chromosomal “secondary constriction,” to be distinguished from the primary constriction or the centromere, was discovered still earlier (Ferguson-Smith et al. 1962).

Nevertheless, the first fragile site in the pedantic sense was observed in a woman who had received multiple X-ray irradiations for eczematous dermatitis and later had borne a malformed child (Dekaban 1965). It was unclear if the fragile site observed on the presumed Chromosome 9 from the woman was linked to the birth of this child. Limited survey of the woman’s family showed normal chromosomes in her abnormal child, her husband, and her father (Dekaban 1965). However, this study raised a question that is still relevant in the research field today. There were two types of chromosome abnormality in the woman’s blood cultures. A relatively more frequent type consisted of a break near the third telomere-proximal portion of the long arm on the presumed Chromosome 9 (24.8%) and a less frequent one involved aberrations such as dicentrics, rings, and deletions on random chromosomes (7.1%). In contrast, her skin culture contained a much lower frequency of breakage on Chromosome 9 (6%), despite a comparable level of other chromosome aberrations (8.8%). Moreover, the break seen in the skin culture occurred at a different location (telomere-distal) on Chromosome 9 from that seen in the blood cultures (telomere-proximal). One theory put forth suggested that the X-ray irradiations had induced a clone of abnormal cells with a telomere-proximal fragile site on Chromosome 9 in the woman’s blood-forming tissues but not in skin tissues. Alternatively, though it could not be ascertained thoroughly due to family members in absentia, there existed tissue-specific formation of chromosome fragile sites. The latter possibility was subsequently more convincingly demonstrated (Kuwano et al. 1990; Murano et al. 1989a). However, the

mechanism of tissue-specific fragile site formation still remains a major challenge in the field.

21.2.2 First Report of a Heritable Fragile Site

The first heritable fragile site was demonstrated in a mother-and-daughter case, which involved a fragile site on the long arm of Chromosome 2 near the centromere (Lejeune et al. 1968). The heritability of this fragile site was later confirmed independently (Ferguson-Smith 1973). Curiously, this fragile site gave rise to duplication of the centromere-distal two-thirds of 2q and formation of a three-armed chromosome, which was referred to as qh(2). It was speculated that this endo-duplication event was the result of interruption of a centromere-originated DNA replication signal by the fragile site. Alternatively, it was thought that qh(2) formation was reminiscent of interstitial telomere element-induced endo-duplications elsewhere (Hsu 1963). It was shown that 2q indeed contains interstitial telomere elements, but this fusion site is distinct from the fragile site band 2q11.2 (Ijdo et al. 1991). Finally, it was also surmised that qh(2) formation was due to chromatid breakage followed by mitotic nondisjunction (Ferguson-Smith 1973). To this date, the mechanism for the duplicated q-arms remains a mystery.

Phenotypically the mother and daughter were both of short stature and had intellectual deficiencies, which may or may not be related to the expression of the fragile site. However, subsequent studies demonstrated that the fragility at 2q11.2, which resides in the presumed cytoband responsible for the formation of qh(2), was observed at a significantly higher frequency in “mentally subnormal” school children (Kahkonen et al. 1986) as well as in patients with schizophrenia (Chen et al. 1998). It was not until recent that this fragile site was molecularly characterized as a site with CGG repeat expansion impacting the *AFF3* gene and was detected in families with a broad range of neurodevelopmental disorders (Metsu et al. 2014a).

21.2.3 First Demonstration of Mendelian Transmission of a Fragile Site

A fragile site on 16q22 was examined in a large family of a boy who had recurrent cold urticaria and immunoglobulin A deficiency (Magenis et al. 1970). Transmission of the fragile site followed a Mendelian pattern. However, later it was demonstrated that this fragile site is distal from the haptoglobin gene whose deficiency is linked to IgA deficiency, suggesting that the IgA condition in the proband was likely a coincidence (Simmers et al. 1986). Fragility at 16q22 has since been implicated in a wide spectrum of neurological disorders (Demirhan et al. 2006; Kerbeshian et al. 2000) as well as in other conditions such as neutropenia (Glasser et al. 2006; Tassano et al. 2010) and cleft palate (Bettex et al. 1998; Dunner et al. 1983; Janiszewska-Olszowska et al. 2013; McKenzie et al. 2002). A recent study also reported elevated fragile site formation at 16q22.1 in an embryo from a couple who had difficulty achieving pregnancy and in the sperm from the father (Martorell et al. 2014). However, cytogenetic breakage and potential disease-associated gene(s) at this locus are yet to be molecularly characterized.

21.2.4 First Fragile Site Linked to a Clinical Phenotype

The vast majority of fragile sites during the early discoveries appeared innocuous and not associated with any phenotypic abnormalities, even when present in homozygous conditions such as those fragile sites at 10q25.2 and 12q24 (Sutherland 1981; Voiculescu et al. 1991). The only fragile site definitively associated with a clinical phenotype was that which resides on Xq27.3 (FRAXA) and was associated with the Martin-Bell or the fragile X syndrome, an X-linked and most common familial form of mental retardation (Giraud et al. 1976; Harvey et al. 1977; Lubs 1969). Subsequent studies demonstrated that the FRAXA fragility is the result of a CGG repeat expansion and is correlated with hypermethylation, gene silencing, as well as delayed replication at the fragile X mental retardation 1 (*FMR1*) gene locus (Bell et al. 1991; Dietrich et al. 1991; Hansen et al. 1992, 1993; Heitz et al. 1991; Pieretti et al. 1991). A full mutation (>200 CGG repeats) at the *FMR1* locus causes gene silencing and the loss of the protein product, FMRP, which causes the loss of synaptic plasticity and the fragile X pathology. Fragile X biology has been the subject of numerous and up-to-date reviews to which we direct the reader for a comprehensive understanding of the disease etiology, mechanism, and intervention (Ligsay and Hagerman 2016; Lozano et al. 2016; Santoro et al. 2012; Wang et al. 2012; Zhao and Usdin 2016).

21.3 Classification of “Common” vs. “Rare” Fragile Sites

21.3.1 Population Frequencies

The Edinburgh survey estimated that from 3% to 5% of the population contained identifiable chromosomal variants (Court Brown 1966). Subsequent population studies defined two types of fragile sites: those that were present in <1% of the general population and those that were present at a theoretical frequency between 1% and 99% or polymorphic (Hecht 1988). Later, this fine distinction between the two groups was relinquished, and collectively they were referred to as “rare fragile sites” with an observed maximal frequency of 5% (Schmid et al. 1986). This classification was also necessitated by the discovery of what appeared to be common or ubiquitous fragile sites, which were present in all 12 subjects regardless of sex or clinical phenotypes (Glover et al. 1984). Thus, presently, fragile sites are classified as “rare” and “common” fragile sites (RFSs and CFSs, respectively) based on the definitions above.

Both RFSs and CFSs can be further characterized by another important metric—the frequency at which fragile sites are observed/expressed in the cells from a given individual, which is akin to a measurement of penetrance. As current literature makes broad reference to frequencies of fragile sites—i.e., at a population level vs. at a cellular level within an individual—it is important to note the difference between these two metrics. For instance, the frequency of FRAXA expression in fragile X individuals can be as high as 50% (Glover et al. 1984). In contrast, the frequency of aphidicolin-induced CFS expression in individuals can be as low as 0.01% based on a large-scale study (Mrasek et al. 2010). Therefore, arguably a CFS with extremely low penetrance may be better characterized as a RFS because it is conceivable that certain individuals, yet to be identified, might exhibit abnormally high penetrance.

21.3.2 Methods of Induction

It was serendipity that led to the discovery that many RFSs discovered by routine diagnostic cytogenetic screen in early studies were unstable in medium 199, which is deficient for folic acid (Sutherland 1977). This finding thus defined the first group of RFSs, which are sensitive to folate stress induced by folic acid or thymidine deprivation, inhibitors of folate metabolism, inhibitors of thymidylate synthetase, and excess thymidine (Sutherland 1991). Systematic analyses revealed that other culture conditions, such as pH, also impact the frequency of fragile site expression (Sutherland 1979). RFSs can also be induced by a group of chemicals of the non-folate stress type such as a nucleotide analog, bromodeoxyuridine, and a base intercalator, distamycin A. In contrast, CFSs are primarily induced by a DNA polymerase inhibitor, aphidicolin, and to a lesser extent by other chemicals including bromode-oxyuridine and 5'-azacytidine.

It is unclear why RFSs and CFSs show drug-specific induction. Genomic loci may be differentially susceptible to drug-induced replication perturbation. This apparent drug specificity of fragile site expression is likely also due to limitations of the cytological screening methods. Consequently, a given fragile site has been traditionally associated with a specific drug though other drugs could also induce its expression, albeit not as potently. Consistent with this notion, the most frequent aphidicolin-induced CFSs have been shown also inducible by thymidylate stress (Kähkönen et al. 1989). Thus, thymidylate stress can induce not only RFSs but also CFSs, demonstrating effectively “cross-induction” of these two classes of fragile sites. The cross-induction of RFSs by aphidicolin was also confirmed by a genomic scale survey (Mrasek et al. 2010). We further asked if there is a correlation between the expression frequencies (percentage of cells showing fragile sites) of seven RFSs when induced by folate stress (Kähkönen et al. 1989) vs. by aphidicolin (Mrasek et al. 2010). For the study by Kähkönen et al., we calculated the expression frequencies only from phenotypically normal individuals for comparison with the study by Mrasek et al. With the exception of a single outlier, FRA22A, which appeared to be highly inducible by aphidicolin, the remaining six RFSs showed a positive correlation ($R = 0.8$) between the induction frequencies by the two conditions (Fig. 21.1). Nevertheless, the differential mechanisms of drug induction of fragile site expression await further exploration.

21.3.3 DNA Sequence and Structural Features

Molecular cloning permitted structural distinction between RFSs and CFSs beyond population frequencies and induction methods. RFSs are characterized by repetitive DNA sequences. Of the 30 RFSs, 12 have so far been identified with a repeat motif with 10 containing a CGG trinucleotide repeat tract and 2 containing an AT-rich minisatellite repeat (Table 21.1). Repetitive DNA can generate non-B DNA structures or alternative conformations with unusual secondary structures (Choi and Majima. 2011). For instance, folate-sensitive RFSs are enriched in CGG repeats that can form stable hairpins, slipped strand structures, G-quadruplexes, and i-tetra-plex structures (Fry and Loeb 1994; Usdin and Woodford 1995; Kang et al. 1995). CGG repeats are capable of pausing or stalling replication forks both in vitro and in vivo (Samadashwily et al. 1997). These repeats are polymorphic in normal individuals but undergo dynamic expansion/contraction, resulting in the cytogenic expression of fragile sites under folate stress (Kremer et al. 1991; Verkerk et

al. 1991). Non-folate-sensitive RFSs on the other hand consist of expandable AT-rich minisatellite repeats and are specifically induced by AT-dinucleotide binding chemicals such as distamycin A and berenil. For instance, the most frequently observed RFS, FRA16B, contains a polymorphic 33 bp AT-rich minisatellite repeat, which can expand to 2000 copies in certain individuals (Yu et al. 1997). Fourteen copies of this 33 bp AT-rich minisatellite repeat were shown to form DNA secondary structures and cause replication fork stalling, fork regression, and polymerase skipping in vitro (Burrow et al. 2010). Similarly, the BrdU-induced non-folate-sensitive RFS, FRA10B, shares homology with the 33 bp minisatellite in FRA16B and contains an 11 bp inverted repeat that can form hairpins (Handt et al. 2000; Yu et al. 1997).

In contrast, CFSs are not characterized by expandable di- or trinucleotide repetitive sequence. However, some of the cloned CFSs are enriched for short interrupted AT-rich islands with high torsional flexibility and high propensity to form stable secondary structures, similar to the RFSs (Dillon et al. 2013; Zlotorynski et al. 2003). Moreover, a study using a yeast-based genetic assay identified a flexibility peak region (Flex1) of FRA16D with a perfect AT/TA repeat element capable of stalling replication forks (Zhang and Freudenreich 2007). Interestingly, these repeats resemble the AT-rich minisatellites found in non-folate RFSs. The extent of stalling depends on the length of the repeat. Therefore, there appears to be a shared mechanism of repeat-based secondary structure formation and perturbation of the replication fork between RFSs and CFSs, the difference being that RFSs have greater expandability than CFSs (Schwartz et al. 2006). Are there any other cis elements, in addition to DNA secondary structures, that define fragile sites? An RNA/DNA hybrid molecule known as R-loop appears to qualify. R-loops play a wide array of functions in normal cells. For instance, R-loops occur in replication origins in mitochondria as primers for DNA replication (Lee and Clayton 1996). R-loops at CpG islands may also facilitate replication origin specification by generating single-stranded DNA in the nuclear genome (Lombrana et al. 2015). In stimulated B-lymphocytes, R-loop formation at the immunoglobulin heavy chain locus facilitates class switch recombination (Yu et al. 2003). Finally, it was shown that R-loop formation in the guanine-rich transcription pause sites downstream of the poly-A signals is required for transcription termination (Skourti-Stathaki et al. 2011). The genome-wide association of GC skew (asymmetry in guanine distribution between the two strands of DNA), which is conducive to R-loop formation, with transcription termination sites was subsequently validated (Ginno et al. 2013). On the flip side, R-loops are increasingly associated with genome instability and human diseases (Groh and Gromak 2014; Santos-Pereira and Aguilera 2015). In yeast, R-loop formation can sensitize DNA to damaging agents, causing double-strand breaks (Huertas and Aguilera 2003; Li and Manley 2005; Sordet et al. 2009). R-loops at transcription termination sites also require proper resolution by factors such as senataxin, and unresolved R-loops can cause genome instability (Hatchi et al. 2015; Skourti-Stathaki et al. 2011). R-loops can also trigger epigenetic changes in the DNA and bring about the formation of repressive chromatin, which in turn could impede DNA replication (Groh et al. 2014). Evidence for blockage of replication fork by R-loop formation was demonstrated for FRA16D (Madireddy et al. 2016). Finally, R-loop formation can endanger genome stability by incurring replication-transcription conflicts, as exemplified by their association with CFS

formation, particularly in large genes such as *FHIT* (Helmrich et al. 2011). The question becomes what roles do R-loops play in replication stress-induced fragile site formation, the corollary being, is there a genome-wide correlation between R-loop content/density and the probability of fragile site formation?

Recent advance in experimental mapping of R-loops capitalized on the utility of the S9.6 antibody, which has a sequence-independent affinity toward the A/B helical RNA/DNA duplex, and coupled the immunoprecipitation of the DNA/RNA hybrid with high-throughput sequencing. Variations of this methodology include DRIPc-seq (DNA/RNA immunoprecipitation followed by cDNA conversion coupled with high-throughput sequencing, an improved version of the previous DRIP-seq method) (Sanz et al. 2016) and RDIP (RNA/DNA immunoprecipitation, implementing key technical modifications in RNase I pretreatment and DNA fragmentation by sonication) (Nadel et al. 2015). DRIPc-seq detected ~70,000 R-loop peaks in the human embryonic carcinoma Ntera2 cells, which is comparable to the number of R-loops detected by RDIP-seq (~64,000 and 39,000 in IMR-90 and HEK 293 T cells, respectively). Consistent with R-loops being co-transcriptional structures, DRIPc-seq peaks were found predominantly in RNA polymerase II-transcribed genes, with two- to three-fold enrichment at the promoter and terminator regions (Sanz et al. 2016). Interestingly, this co-transcriptional model of R-loop formation was contended by the RDIP-seq study, at least at the ribosomal DNA loci (Nadel et al. 2015). Moreover, using a more sensitive readout (than RNA-seq) for transcription, the global run-on sequencing (GRO-seq) method, it was found that only 47.7% of the RNA/DNA hybrids are associated with active transcription (Nadel et al. 2015). Finally, the RDIP-seq study reported a moderate depletion of R-loops at the terminators, in contrast to a twofold enrichment at the promoters (included in a 1.5 kb window downstream from the transcription start site) (Nadel et al. 2015). These studies highlighted the dynamic nature of R-loops and their potential cell type-specific formation. They also necessitate an unbiased approach to identify R-loops in the human genome.

Sequences with high CG content are more conducive to R-loop formation due to high thermostability of the rG-C base pair (Roy and Lieber 2009; Sugimoto et al. 1995; Tracy et al. 2000). Training on previously discovered R-loops, a quantitative model of R-loop forming sequences (RLFSs) has been developed and used to predict RLFSs in the known transcribed regions of the human genome (Wongsurawat et al. 2012). Using the R-loop database (<http://rloop.bii.a-star.edu.sg/>), we tallied the number of R-loops in each gene of the RFSs as well as calculated their density (number of R-loops per kb in a given gene), as reported in Table 21.1. Our analysis shows that genes residing in RFSs have relatively higher concentration of R-loop forming sequences compared to all genes (Table 21.3). We also queried which RFSs and genes have the highest R-loop content (top 1%), either based on total number or density (Fig. 21.2). The analysis reveals that FRA22A is enriched for genes with the highest number of R-loops (more than 70 R-loops per gene) and that FRA19B is enriched for genes with the highest R-loop density (more than 2.5 R-loops per kb sequence). High R-loop content, together with the fact that many RFSs contain CGG repeat, is intriguing. Because the computational model for R-loop prediction defines an R-loop core sequence as clusters of 3–4 contiguous Gs interspersed by a single nucleotide, CGG trinucleotide repeats per se are not R-loop forming sequences. It would be interesting to test

if these two entities have coevolved and that the higher than average R-loop content in the RFSs predisposes CGG repeats to break.

We performed similar analysis with a subset ($n = 11$) of CFSs, which have been cloned, and the associated genes identified (13 total, with FRA10G and FRA10G each housing two genes): FRA1B(*DABI*), FRA2F(*LRP1B*), FRA3B(*FHIT*), FRA4F(*GRID2*), FRA6E(*PARK2*), FRA10D(*CTNNA3*), FRA10G(*RET*, *NCOA4*), FRA15A(*RORA*), FRA16D(*WWOX*), and FRA10G(*DMD*, *IL1RAPL1*). Seven of these 13 genes (54%) contain 11–50 R-loops, which appeared significantly higher than the reported genomic average (5%) with the same level of R-loop content (Table 21.3). Thus, R-loop formation might also be associated with CFSs, consistent with previous findings that breakage at CFSs harboring large genes can be due to R-loop formation deterring transcription complexes (Helmrich et al. 2011; Wilson et al. 2015).

21.3.4 Mechanisms of Fragile Site Formation

Early studies observed that the fragile X chromosome showed delayed or incomplete replication due to the slowing down or stalling of replication forks (Hansen et al. 1993). This study presented an attractive model for fragile site development as approximately 1% and 20% of the genome in transformed and non-transformed human cells, respectively, undergoes replication even during mitosis (Widrow et al. 1998). Subsequent studies also confirmed late replication timing of a handful of CFSs (Handt et al. 2000; Hellman et al. 2000; Le Beau et al. 1998; Palakodeti et al. 2004; Pelliccia et al. 2008; Wang et al. 1999). However, not all fragile sites appear to replicate late—some are located in the interface of early- and late-replicating regions (El Achkar et al. 2005; Handt et al. 2000), and others are in fact associated with early replication timing (Barlow et al. 2013). Besides replication timing anomalies, CFSs can also be sites of collisions between the replication and the transcription machineries (Helmrich et al. 2011). Additionally, defective processing of CFSs in a Rad52-dependent DNA repair pathway has also been proposed to contribute to fragile site instability (Bhowmick et al. 2016; Sotiriou et al. 2016). However, the requirement for DNA repair is likely linked to a potential underlying defect in DNA replication. Therefore, there appears to be two major factors that underlie fragile site instability: defective DNA initiation/progression and replication-transcription conflicts (Le Tallec et al. 2014; Ozeri-Galai et al. 2014; Sarni and Kerem 2016). Here, we highlight two major areas with competing models, one regarding the replication defects at fragile sites and the other pertaining to the impact of transcriptional status of large genes on chromosome fragility.

While a strong correlation exists between DNA secondary structure formation and fork stalling in both micro- and minisatellites of RFSs, evidences for such direct implication for instability in CFSs are contradictory. Is chromosome fragility the consequence of replication fork stalling at AT-rich sequences in CFSs? Previous studies have shown that replication across CFSs is delayed compared to other regions of the genome, particularly in the presence of aphidicolin (Hellman et al. 2000; Le Beau et al. 1998; Palakodeti et al. 2004; Wang et al. 1999). However, recent studies employing DNA combing have countered that replication fork speed is not different between fragile sites (FRA3B and FRA6E) and non-fragile regions, with or without aphidicolin treatment (Letessier et al. 2011; Palumbo et al.

2010). It was further shown that fragility at FRA3B is the consequence of deficient origin activation within the fragile site when replication fork is slowed down by aphidicolin (Letessier et al. 2011). In contrast, using a similar DNA combing approach, it was shown that replication forks indeed stall at AT-rich sequences in FRA16C (Ozeri-Galai et al. 2011). These contradictory results can be partly explained by significant differences in experimental conditions for replication fork rate measurement, such as the duration of pulse labeling by nucleoside analogs (in DNA combing) and aphidicolin dosage. Considering that fragile site expression level varies significantly with aphidicolin concentrations (Glover et al. 1984), direct comparison between fork rates measured under such different conditions is tenuous. The fact that FRA16C coincides with a RFS, FRA16B, further precludes direct comparison between these studies. Thus, future studies will undoubtedly benefit from standardized experimental conditions. However, both studies agree that paucity of origins in the fragile site region is at least partially responsible for the delayed replication completion of fragile sites (Letessier et al. 2011; Ozeri-Galai et al. 2011; Palumbo et al. 2010).

Another contentious topic is whether the transcriptional status of a large gene impacts fragile site formation. Helmrich et al. suggested that collision between replication and transcription at large genes causes chromosome fragility and, further, breakage frequency is correlated with gene expression level (Helmrich et al. 2012). The authors further posited that R-loop formation as a consequence of transcription along the fragile locus could pose a serious obstacle to the replication fork. However, Le Tallec et al. argued that the expression level of large genes does not correlate with chromosome fragility (Le Tallec et al. 2013). Again, this apparent discrepancy between the two studies is at least attributable to multiple differences in experimental conditions including aphidicolin concentration, definition of large genes, and calculation of break frequency. It further underscores the importance of applying standardized or comparable experimental conditions for effective comparisons between studies.

Finally, an altered epigenetic environment in the fragile loci could be another cause for fragile site instability. The inability to undergo condensation at the time of mitosis could result in DNA strand breakage. Using an in vitro nucleosome reconstitution assay, it was shown that CGG repeats with greater than 50 copies exclude nucleosome and that this exclusion is dependent on the length of the repeat (Wang and Griffith 1996; Wang et al. 1996). It was also shown that the expanded AT-rich minisatellites in FRA16B can exclude nucleosome assembly albeit only in the presence of distamycin A (Hsu and Wang 2002). Finally, it has been shown that hypo-acetylation occurs in CFSs compared to genomic average, indicating a compact chromatin around CFSs (Koch et al. 2007; Savelyeva and Brueckner 2014).

21.3.5 Disease Associations

Fragile sites have been associated with both genetic and epigenetic instability (Smith et al. 2010). Currently there are 11 RFSs that have been molecularly mapped, i.e., the gene(s) that are impacted by fragile site formation have been identified. Many of them are associated with a definitive human disease, predominantly a neurological disorder (Table 21.1). Interestingly, folate-sensitive RFSs are specifically associated with neuropsychiatric

disorders including schizophrenia, autism spectrum disorders (ASDs), and mental retardation, while non-folate-sensitive RFSs are associated with a more diverse set of disorders such as infertility and Langer-Giedion syndrome. Schizophrenia, ASDs, and bipolar disorders are all complex neurological disorders that have intricate association with genetics and the environment (Kerner 2014; Miles 2011; Smith et al. 2010). No single gene can account for all the symptoms that characterize these diseases. Therefore, it stands to reason that the genes impacted by chromosome fragility at the folate-sensitive RFSs have a global impact on gene expression and/or protein production in the brain. Many of the CGG repeat-containing genes indeed have high expression in the brain (*AFF3*, *ZNF713*, *FAM10A1*, *FMR1*, *FMR2*) (Uhlen et al. 2010). As the biochemical functions of these RFS-impacted genes become clear, it will help us understand the disease etiology in each of the associated diseases. For instance, the protein product of *FMR1*, FMRP, is an RNA-binding protein and is estimated to bind 4% of the mRNAs in the brain and regulate their translation (Santoro et al. 2012). Therefore, it remains a challenge to fully understand the genomic impact of FMRP deficiency in the fragile X syndrome.

Moreover, differential expression levels of the disease-associated genes can cause different diseases. For instance, in the case of the *FMR1* gene at FRAXA, a full mutation (>200 CGG repeats) induces *FMR1* gene silencing and results in the fragile X syndrome, while premutation alleles (55–200 CGG repeats) cause fragile X-associated ataxia/tremor syndrome and fragile X-associated primary ovarian insufficiency [note: premutation alleles do not cause chromosome fragility] (Galloway and Nelson 2009; Garcia-Arocena and Hagerman 2010; Santoro et al. 2012; Usdin et al. 2014). These observations suggest that impaired function of the RFS-associated genes can also impact organs other than the brain. The aforementioned brain-expressed genes associated with RFSs also show high expression in the reproductive organs (Uhlen et al. 2010). In addition, *FAM11A* gene expression is high in bone marrow, nervous system, and endocrine glands (Unger et al. 2013). Finally, there seems to be a dominance of neurological disorders, as opposed to cancer, associated with RFSs for unknown reasons. *CBL2* is the only CGG repeat-associated gene that is both seen in neurological disorders and cancer—while CGG repeat expansion and fragility at *CBL2* are linked to Jacobsen syndrome (Jones et al. 1995), *CBL2* has also been reported as a proto-oncogene associated with cancer breakpoints in several forms of leukemia and lymphomas (Fu et al. 2003).

In contrast, CFSs are clearly associated with cancer breakpoints and their expression linked to carcinogenesis (Arlt et al. 2006; Ma et al. 2012). Interestingly, some CFSs are also implicated in neurological disorders (Parkinson's, schizophrenia, and intellectual disability) and a variety of diseases associated with immunodeficiency, bone disorders, and infertility (Table 21.2). Twenty-eight of the 90 CFSs have genes associated with schizophrenia (Smith et al. 2010). Two CFSs, FRA7H and FRA7F, also showed high expression in cells from bipolar disorder patients compared to normal individuals when induced with folate-deficient media (Demirhan et al. 2009). These studies indicate that CFSs may trigger neurological symptoms more frequently than previously thought. The latter study also highlights the importance of understanding the cross-induction of fragile sites by chemicals and growth conditions.

Molecular cloning has enabled the identification of disease-associated gene(s) within a broadly defined fragile site. As shown above, R-loops are enriched in both RFSs and CFSs compared to genomic average. R-loops have been associated with neurological disorders such as the dominant juvenile form of amyotrophic lateral sclerosis type 4 (*ALS4*) and a recessive form of ataxia oculomotor apraxia type 2 (*AOA2*) (Chen et al. 2004; Moreira et al. 2004). A firm link between R-loop and oncogenesis was also established when it was shown that *BRCA2*, mutated in breast and ovarian cancer, is required to prevent R-loop accumulation and genome instability (Bhatia et al. 2014). We speculate that RLFSs can serve as a marker for disease-associated genes. Among the genes residing in RFSs, *GALNT9* contains the highest number (315) of R-loops (Fig. 21.2). It has been shown that *GALNT9* is frequently methylated and silenced in breast to brain metastasis (Pangeni et al. 2015) and its gene expression is a prognostic marker in neuroblastoma patients (Berois et al. 2013). Whether the high R-loop content plays a role in epigenetic regulation of *GALNT9* is an interesting question that warrants further investigation. Similarly, *CTAG2* shows the highest R-loop density (5 per kb sequence) among RFS-associated genes (Fig. 21.2). In CFSs, the *RET* gene at FRA10G has the highest number of R-loops and the highest R-loop density (1 every 1.6 kb, Fig. 21.2). *RET* rearrangements have been observed in several cancerous cell lines (Dillon et al. 2010). Undoubtedly, systematic analysis of R-loop distribution in all known CFSs would be vitally important.

21.4 Genome-Wide Mapping and Analysis of Fragile Sites

Many of the questions regarding population frequency and penetrance of fragile sites can be more effectively addressed by studies with increased scale and resolution. For instance, are there multiple breakage hot spots in a fragile site? How do the breakage spectra vary between cell types and inducing drugs? Mrasek et al. systematically identified aphidicolin-induced CFSs by screening 25,000 metaphase chromosome spreads isolated from lymphocytes of three normal and unrelated individuals (Mrasek et al. 2010). As alluded to above, this study demonstrated that the classically defined RFSs are less dependent on folate stress for induction than previously considered—all but 3 (FRA6A, FRA12D, and FRAXA) of the 30 RFSs were induced by aphidicolin, and only 4 were present in fewer than 3 individuals (total frequency among 3 individuals for each site listed in Table 21.1). However, based on our analysis of the data reported by Mrasek et al., the penetrance of RFS when induced by aphidicolin is significantly lower than that of CFSs, with the median levels being 0.075% and 0.464%, respectively, confirming the biased potency of aphidicolin toward CFSs.

Cytogenetic screens, while discernible and powerful, are not amenable for genome-wide and high-resolution identification of fragile sites. The advent of deep sequencing technology now permits fragile site mapping at a global scale and a faster pace. Currently the following methods have been applied to map DNA double-strand breaks (DSBs) in the human genome: BLESS (direct in situ break labeling, enrichment on streptavidin, and next-generation sequencing) (Crosetto et al. 2013), RAFT (rapid amplification of forum termini involving direct ligation of biotinylated oligonucleotides to DNA DSBs) (Tchurikov et al. 2015), and DSB-seq (using terminal deoxyribonucleotidyl transferase labeling of DSBs with biotinylated nucleotides followed by streptavidin pull-down and library construction)

(Baranello et al. 2014). However, only one study applied conditions to induce CFSs, and it identified over 2429 aphidicolin-induced DSBs after correction for copy number variation in HeLa cells (Crosetto et al. 2013). The authors stated “many CFSs were scored as sensitive to aphidicolin following our approach.” Based on our analysis, 190 (8%) and 574 (24%) of these 2429 DSBs overlap with the known rare and common fragile site regions (defined as cytoband coordinates from the UCSC Human Genome Database), respectively. The apparent lack of correlation for these aphidicolin-induced DSBs with RFSs is not unexpected. But the moderate level of overlap with CFSs begs discussion, and we attribute it to the following reasons. First, the moderate level of concordance is most likely the result of cell type (HeLa)-specific fragile site expression with fragile sites primarily defined in lymphocytes. Second, it also highlights the fundamental difference between methodologies. On one hand, cytologically defined chromosome breakage might include also single-stranded DNA breakage which would evade detection by BLESS. On the other hand, computationally predicted micro-fragile chromosomal regions might be missed by cytological screening (Thys et al. 2015). Finally, the usage of growth medium and concentrations of inducing drugs, e.g., aphidicolin, are not standardized across different studies and impinge on fragile site formation. For these reasons, it is at once a necessity and a challenge for future genomic mapping of fragile sites to compare different cell types with standardized inducing conditions.

21.5 Unsolved Mysteries of Chromosome Fragile Sites

So far, we have discussed some of the contentious topics in chromosome fragility in sections above. In this section we highlight three phenomena that still confound researchers and remain not understood. We also note that additional mysteries previously articulated in “Forgotten fragile sites and related phenomena” still remain unsolved (Sutherland and Baker 2003).

21.5.1 What Is the Underlying Cause for Tissue-Specific Fragile Site Formation?

The very first reported case of fragile site already noted cell type-dependent fragile site expression as discussed earlier in this document. Mounting evidence further confirmed this observation (Hosseini et al. 2013; Kuwano et al. 1990; Le Tallec et al. 2013; Letessier et al. 2011; Murano et al. 1989a, 1989b). Since chromosome fragility is enhanced by replication fork instability, the varying locations and activation patterns of origins of replication across tissue types would directly impact fragile site expression. In addition, tissue-specific gene expression would also influence sites of replication-transcription conflict-induced chromosome fragility. Related to this latter point, we have recently postulated that the inducing agent for fragile site expression plays a dual role in simultaneously generating replication stress and untimely gene expression while replication is still incomplete, resulting in replication-transcription conflicts at distinct loci in different cells (Hoffman et al. 2015). This hypothesis was derived from the model organism *Saccharomyces cerevisiae*, and we are currently testing it in human cell lines. Future studies mapping fragile sites in different cell lines with simultaneous measurements of origin activities and gene expression levels will be ideal for understanding tissue-specific fragility.

21.5.2 Why Are RFSs Preferentially Induced by Folate Stress?

Perhaps one of the foremost interesting questions is how do different classes of drugs define fragile sites. For instance, folic acid deprivation, thymidylate synthase inhibition (e.g., fluorodeoxyuridine), and thymidine deprivation or excess can all induce a class of folate-sensitive RFSs. One of the outstanding features of the folate-sensitive fragile sites is that nearly half of them (10 out of 22) have been found to contain CGG repeats thus far. Does folate deficiency preferentially lead to DNA breaks at CGG repeats? Folic acid is crucial for methyl metabolism, which in turn impacts DNA replication and repair. Deprivation of folic acid blocks the methylation of dUMP to TMP and triggers an increase in dUTP level. Similarly, intra-cellular fluorodeoxyuridine is converted to fluorodeoxyuridine monophosphate, which in turn inhibits thymidylate synthase and also causes an increase of dUTP. Consequently, there is an increase in the incorporation of uracil into DNA. Uracil in DNA is removed by the uracil DNA glycosylase (UDG), which, through a facilitated diffusion mechanism, locates the damaged/modified DNA bases (Schonhofs et al. 2013). It has been proposed that folate-sensitive chromatid breakage is the result of a catastrophic DNA repair cycle where excision of uracil is followed by reincorporation of uracil due to continuous blockage of the dTTP pool (Reidy 1987).

The question then becomes is there a higher level of uracil incorporation occurring at CGG repeat regions than other chromosomal regions, or are CGG repeats more conducive to uracil excision? There seemed to be evidence supporting both arguments. A genome-wide study in *S. cerevisiae* has demonstrated that the uracil content is relatively lower in early-replicating regions than late-replicating ones (Bryan et al. 2014). RFSs such as FRAXA tend to be late-replicating (Hansen et al. 1993; Subramanian et al. 1996; Webb 1992) and therefore might incorporate uracil at a higher rate. Alternatively but not mutually exclusively, cytosine deamination to uracil at the CGG repeats might be an underlying cause for increased uracil content. On the other hand, incorporated uracil might be easier to recognize by the UDG enzyme in the context of secondary structures due to CGG repeats. Supporting this hypothesis is the observation that a related DNA glycosylase, the human alkyladenine DNA glycosylase, can capture the site of DNA damage more efficiently on a flexible DNA template containing kinks, bubbles, or gaps than on a continuous B-form DNA duplex (Hedglin et al. 2015). Conceivably, this characteristic can also extend to UDG acting on a DNA template enriched for CGG repeats which readily form hairpin structures.

21.5.3 What Is the Underlying Cause for Sex-Biased Transmission of Autosomal Fragile Sites?

Sherman and Sutherland reported in a population study that folate-sensitive and BrdU-sensitive autosomal fragile site expression was higher when the carrier parent was the mother than if it was the father (Sherman and Sutherland 1986). Similarly, Kähkönen et al. also observed that there was a maternal bias of the transmission of autosomal RFSs (16 out of 19 families were maternal carriers and 1 was a paternal carrier) (Kähkönen et al. 1989). This phenomenon was once again reported much later in a study demonstrating predominantly maternal transmission of ring chromosome 15 (Nikitina et al. 2003). What is the underlying cause for this sex-biased fragile site inheritance? Is the fragile site expression dependent on genomic imprinting of the maternal genes? Or is the maternal transmission of

fragile sites a consequence of nonrandom chromosome arrangement in germ lines? Finally, is this phenomenon unique to folate stress? Large-scale population studies are required to confirm these findings. Further genetic and epigenetic characterizations of the autosomal RFSs will also shed new light on this genetic puzzle.

Acknowledgments

We thank Andrew McCulley for assisting data analysis during this work. This work was supported by the National Institutes of Health (5R01 GM118799-01A1 to W.F.).

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Fig. 21.1. Comparison between *RFS* expression frequencies induced by folate stress and by aphidicolin (APH). Experimental values obtained from Kähkönen et al. (1989) and Mrasek et al. (2010), respectively

Fig. 21.2.

Probability plots showing the distribution of 1973 genes within *RFSs* by the number of *RLF*s per gene (**A**) or by the number of *RLF*s per kb for a given gene (**B**). The top 1% of genes in each plot are as shown

Table 21.1

Updated summary of *RFSSs*

Fragile site	Type	Repeat	Cyto-band	Population freq. (%) ^d	Size (kb)	No. of <i>RLFSSs</i>	No. of <i>RLFSSs</i> /kb	No. of genes with > = 1 <i>RLFSS</i> /kb	Freq. (%) in folate stress ^d	Freq. (%) in <i>ApoB</i> ^d	Assoc. genes	Disease
FRA1M	Folate		1p21.3		4,900	48	0.010	0		3.333		ID (D' Angelo et al., 2015)
FRA2A	Folate	CGG	2q11.2	0.0014	6,400	266	0.042	1	27.00	0.089	<i>AFF3</i>	Schizophrenia (Chen et al. 1998); ID (Metsu et al. 2014a)
FRA2B	Folate		2q13		5,200	177	0.034	0		0.067		
FRA2K	Folate		2q22.3		3,700	20	0.005	0		0.626		ID (Mulatinho et al. 2012)
FRA3G	Folate		5q35		13,458	898	0.067	6		0.120		Found in a FX patient at 50% expression and a normal brother at 36% (Howell et al. 1990)
FRA6A	Folate		6p23		2,000	99	0.050	1		0.009 <i>C</i>		Schizophrenia (Olavesen et al. 1995)
FRA7A	Folate	CGG	7p11.2		3,500	89	0.025	1		0.058	<i>ZNF713</i>	ASD (Metsu et al. 2014b)
FRA8A	Folate		8q22.3		4,500	164	0.036	2		0.018		
FRA9A	Folate		9p21	0.0041	12,900	50	0.004	0	21.00	0.102		
FRA9B	Folate		9q32		2,800	136	0.049	0		1.513		
FRA10A	Folate	CGG	10q23.3	0.0028	16,707	168	0.010	0	35.67	0.124	<i>FRA10AC1</i>	Single case of tumor syndrome (Li et al. 2015; Malby and Higgins 1987); Alzheimer's (Li et al. 2015)
FRA11A	Folate	CGG	11q13.3		1,500	165	0.110	2		0.075	<i>C11orf8c/MIP1D2</i>	
FRA11B	Folate	CGG	11q23.3		5,300	459	0.087	1		0.098	<i>CBL2</i>	Jacobsen syndrome (Jones et al. 1994, 1995; Michaelis et al. 1998)
FRA12A	Folate	CGG	12q13.1	0.0007	4,678	382	0.082	8	17.67	0.075	<i>DIP2B</i>	ASD and MR (Winnepeninckx et al. 2007)
FRA12D	Folate		12q24.13		2,000	93	0.047	0		Not induced		Segregating in FX families (Amarose et al. 1987; Bartlett et al. 1991; Sutherland and Baker 1993)
FRA16A	Folate	CGG	16p13.11	0.0007	2,000	116	0.058	1	12.00	0.053	N.D.	
FRA19B	Folate		19p13		19,800	4,650	0.235	88		0.036		
FRA20A	Folate		20p11.23		3,400	70	0.021	0		0.027 <i>C</i>		
FRA22A	Folate		22q13	0.0014	13,791	2,445	0.177	17	5.67	0.328		MR (Webb and Thake 1984)
FRAXA	Folate	CGG	Xq27.3		5,000	17	0.003	0		Not induced	<i>FMR1, ASFMR1/FMR4</i>	FXS, MR, ID, FXTAS, FXPOI (Galloway and Nelson 2009; Santoro et al. 2012; Usdin et al. 2014)
FRAXE	Folate	CGG	Xq28		8,014	615	0.077	13		0.018 <i>C</i>	<i>AFF2/FMR2</i>	MR (Bensaid et al. 2009)
FRAXF	Folate	CGG	Xq28		8,014	615	0.077	13		0.018 <i>C</i>	<i>FAM11A</i>	MR; ASD; psychosis, pyramidal signs, macrocardism (Holden et al. 1996; Lindsay et al. 1996; Viana-Morgante et al. 1996)
FRAE	DA		8q24.1		28,526	1,394	0.049	24		0.799		Azoospermia (Seki et al. 1992); hereditary multiple exostoses 1 gene and two overlapping Langer-Giedion syndrome deletion endpoints (Hill et al. 1997)
FRA11I	DA		11p15.1		5,609	175	0.031	1		0.510		Azoospermia (Seki et al. 1992)
FRA16B	DA	AT-rich MS	16q22.1		4,200	491	0.117	9		0.488	N.D.	Difficulty achieving pregnancy (Martorell et al. 2014); dominant inheritance of cleft palate, microstomia, and micrognathia (McKenzie et al. 2002)
FRA16E	DA		16p12.1		5,900	178	0.030	1		0.027		Batten disease (common form of neuronal ceroid lipofuscinoses [NCLs]) (Dooley et al. 1994)
FRA17A	DA		17p12	0.0049	4,700	91	0.019	2	20.00	0.049		Azoospermia (Shabui et al. 1982)
FRA10B	BrdU	AT-rich MS	10q25.2		3,100	45	0.015	0		0.191	N.D.	

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Fragile site	Type	Repeat	Cyto-band	Population freq. (%) ^a	Size (kb)	No. of RLFSs	No. of RLFSs/kb	No. of genes with > = 1 RLFS/kb	Freq. (%) in folate stress ^d	Freq. (%) in APT ^b	Assoc. genes	Disease
FRA12C	BdU		12q24		24.850	1.907	0.077	6		0.288		Segregating in FX families (Amarose et al. 1987; Barletta et al. 1991; Sutherland and Baker 1993)
FRA8F	UC		8q13		7.900	104	0.013	0		0.004 ^c		

Folate folate stress, *DA* distamycin A, *BrdU* bromodeoxyuridine, *UC* unclassified, *MS* minisatellite, *RLFS* R-loop forming sequence, *ID* intellectual disability, *ASD* autism spectrum disorder, *MR* mental retardation, *FXTAS* fragile X-associated tremor/ataxia syndrome, *FXPOI* fragile X-associated premature ovarian insufficiency, *Freq.* frequency of fragile site expression

^aData derived from Kähkönen et al. (1989)

^bData derived from Mrasek et al. (2010)

^cFragile site detected in fewer than three individuals when induced by aphidicolin

Table 21.2

Summary of *CFS*-associated genes and their implications in human diseases

Fragile site	Induction method	Cyto-band	Freq. in APH (%) ^a	Assoc. genes	Disease
FRA1A	APH	1p36	0.488		
FRA1B*	APH	1p32	1.491	<i>DAB1</i>	Brain and endometrial cancer (McAvoy et al. 2008)
FRA1C	APH	1p31.2		<i>IL23R-C1orf141</i>	Vogt-Koyanagi-Harada syndrome; LOH/MSI associated with advanced tumor stage in prostate cancer (Brys et al. 2013; Hou et al. 2014)
FRA1D	APH	1p22	0.009		
FRA1E*	APH	1p21.2	0.067	<i>DPYD</i>	Colorectal, breast, and ovarian cancer (Hormozian et al. 2007)
FRA1F	APH	1q21	0.098		
FRA1G	APH	1q25	0.799		
FRA1I	APH	1q44	2.299		Nasopharyngeal carcinoma (Xia et al. 1988)
FRA1K	APH	1q31	0.138		
FRA1L	APH	1p31	0.479		
FRA2C*	APH	2p24.2 2q24.3	1.513	<i>MYCN</i>	Two FSs, FRA2Ccen and FRA2Ctel, flank the <i>MYCN</i> amplicon; locus-specific and genomic rearrangements in neuroblastoma and multiple cancers (Blumrich et al. 2011; Lipska et al. 2013)
FRA2D	APH	2p16.2	2.325		
FRA2E	APH	2p13	0.235		Posttransplant diffuse large B-cell lymphoma (Rinaldi et al. 2010)
FRA2F	APH	2q21.3	0.994	<i>LRP1B</i>	Alzheimer's; Kawasaki disease (Lin et al. 2014; Shang et al. 2015)
FRA2G*	APH	2q31	0.306	<i>GADI</i>	Schizophrenia (Bharadwaj et al. 2013)
FRA2H*	APH	2q32.1	3.905	<i>DIRC1, PMS1, MIRN589, MIRN1245</i>	Breakpoints characterized in K562 cells (Pelliccia et al. 2010)
FRA2I	APH	2q33	1.722		
FRA2J	APH	2q37.3	0.657		
FRA3A	APH	3p24.2	1.327		
FRA3B*	APH	3p14.2	14.153	<i>FHIT</i>	Loss or change of <i>FHIT</i> expression associated with common cancers including bladder, esophageal, lung, breast, and prostate cancers (Saldivar et al. 2010)
FRA3C	APH	3q27	1.061		
FRA3D	APH	3q25	0.546		FS expression linked to schizophrenia (Demirhan et al. 2006)
FRA4A	APH	4p16.1	0.612		

Fragile site	Induction method	Cyto-band	Freq. in APH (%) ^a	Assoc. genes	Disease
FRA4C	APH	4q31.1	2.175		
FRA4D	APH	4p15	0.111		
FRA4F*	APH	4q22	0.683	<i>GRD2</i>	Autosomal recessive cerebellar ataxia associated with retinal dystrophy; ataxia and tonic upgaze (Hills et al. 2013; Van Schil et al. 2015)
FRA5C	APH	5q31.1	0.373	<i>SMAD5</i>	Copy number gain and increased expression in human hepatocellular carcinoma cells (Zimonjic et al. 2003)
FRA5D	APH	5q15	0.834		Concomitant t(5;21)(q15;q22) and del(5)(q13q33) events in myelodysplastic syndrome (Kasi Loknath Kumar et al. 2016)
FRA5E	APH	5p14	0.293		Copy number loss observed at 52.4% in early Barrett's esophagus (Lai et al. 2010)
FRA5F	APH	5q21	0.515		FS expression significantly higher in patients with rectum cancer and their first-degree relatives (Tunca et al. 2000)
FRA6B	APH	6p25.1	0.985		Copy number change associated with osteoporotic fractures, gyral pattern anomaly, and speech and language disorder in two dizygotic twins (Bozza et al. 2013; Oei et al. 2014)
FRA6C	APH	6p22.2	0.062	BAC CITB.564_C_7	High LOH in cervical tumors; HPV integration site in cervical tumor CC171 (Rader et al. 1996; Thorland et al. 2000)
FRA6E*	APH	6q26	2.823	<i>PARK2</i>	Parkinson's; poor outcome in breast cancer (Ambroziak et al. 2015; Letessier et al. 2007)
FRA6F*	APH	6q21	0.608	<i>REV3L, DIF13, FKHL, etc.</i>	Cancer; schizophrenia (Karayianni et al. 1999; Morelli et al. 2002)
FRA6G	APH	6q15	0.115		Chromosome breakpoints in metastatic melanoma (Limon et al. 1988)
FRA7B*	APH	7p22	0.799	<i>THSD7A, SDK1, MAD1L1, MIRN589, MIRN339</i>	Recurrent breakpoint in multiple cancers (Bosco et al. 2010)
FRA7C	APH	7p14.2	0.541		
FRA7D	APH	7p13	0.55		Highly expressed in a female presenting severe immunodeficiency (Conley et al. 1986)
FRA7E*	APH	7q21.2	0.501		
FRA7F	APH	7q22	0.107		Fragile site expression linked to bipolar disorder and schizophrenia (Demirhan et al. 2006, 2009)
FRA7G*	APH	7q31.2	0.111	Caveolin-1, caveolin-2, <i>TESTIN</i>	Reduced <i>TESTIN</i> expression in 22% of cancer cell lines and 44% of the cell lines derived from hematological malignancies; caveolin-1 gene proposed to be a candidate tumor suppressor (Engelman et al. 1998; Tatarelli et al. 2000)
FRA7H*	APH	7q32.3	2.374		Translocation involving 7q32 found in the RC-K8 cell line derived from a patient with terminal diffuse large B-cell lymphoma (Mishmar et al. 1998; Schneider et al. 2008)
FRA7I*	APH	7q36	0.084	<i>CNTNAP2</i>	Implicated in ASD (Rodenhas-Cuadrado et al. 2014)

Fragile site	Induction method	Cyto-band	Freq. in APH (%) ^a	Assoc. genes	Disease
FRA7J	APH	7q11	0.83	<i>LIMK1, EIF4H(WBSCR1)</i>	Breakpoint found between the <i>LIMK1</i> and <i>EIF4H (WBSCR1)</i> genes in patients with Williams-Beuren syndrome and ASD (Piaja et al. 2015)
FRA8B	APH	8q22.1	0.923		
FRA8C*	APH	8q24.1	0.799	<i>MYC</i>	Cluster of HPV18 integrations at 8q24 in primary cervical carcinoma; CFS expression frequent in bladder cancer (Ferber et al. 2004; Moriarty and Webster 2003)
FRA8D	APH	8q24.3	0.182		
FRA9D	APH	9q22.1	0.231		FS expression frequent in bladder cancer (Moriarty and Webster 2003)
FRA9E*	APH	9q32		<i>PAPPA</i>	LOH, particularly loss of <i>PAPPA</i> , is linked to ovarian cancer (Callahan et al. 2003)
FRA10D	APH	10q22.1	0.306	<i>CTNNA3</i>	Decreased gene expression of <i>CTNNA3</i> in oropharyngeal squamous cell carcinomas (Gao et al. 2014)
FRA10E	APH	10q25.2	0.191		
FRA10F*	APH	10q26.1	0.408	<i>FATS</i>	
FRA10G*	APH	10q11.2	0.142	<i>RET, NCOA4</i>	FS generates oncogenic <i>RET/PTC</i> rearrangements in human thyroid cells (Gandhi et al. 2010)
FRA11C	APH	11p15.1	0.51		FS expression frequent in bladder cancer (Moriarty and Webster 2003)
FRA11D	APH	11p14.2	0.928		
FRA11E	APH	11p13	0.621		Translocation breakpoints at 11p13 found in CML patients.
FRA11F	APH	11q14.2	1.771		Gene amplification linked to FRA11F expression in oral cancer (Reshmi et al. 2007)
FRA11G*	APH	11q23.3	0.098		Interstitial deletions at 11q23.3 associated with abnormal ultrasound findings during prenatal diagnosis (Fechter et al. 2007; Liu et al. 2014)
FRA11H*	APH	11q13	0.075		
FRA12B	APH	12q21.3	0.364		
FRA12E	APH	12q24	0.288		
FRA13A*	APH	13q13.2	1.061	<i>NBEA</i>	Neuropsychiatric disorders (Savelyeva et al. 2006a, b)
FRA13C	APH	13q21.2	0.124		HPV16 integration site in the cervical tumor cell line SiHa (Thorland et al. 2000)
FRA13D	APH	13q32	0.178		Fragile site expression linked to bipolar disorder and schizophrenia (Demirhan et al. 2006, 2009)
FRA14B	APH	14q23	1.429		Found as a constant FS in bloom syndrome cell lines (Barbi et al. 1984; Shiraishi and Li 1993)
FRA14C	APH	14q24.1	0.382		Found as frequent HPV integration site in HPV-related cancers (Bodelon et al. 2016)
FRA15A	APH	15q22	0.08	<i>RORA</i>	<i>RORA</i> gene expression is low in breast, prostate, and ovarian cancers (Zhu et al. 2006)

Fragile site	Induction method	Cyto-band	Freq. in APH (%) ^a	Assoc. genes	Disease
FRA16C	APH	16q22.1	0.488		High expression at FRA16B/C found in peripheral blood lymphocytes in the male of a couple having trouble conceiving. Chr 16 instability detected in the sperm of the male and in embryos (Martorell et al. 2014)
FRA16D*	APH	16q23.2	7.576	<i>WWOX/WOX1/FOR</i>	<i>WWOX</i> gene dysregulation associated with multiple cancers, including pancreatic adenocarcinoma, renal cell carcinoma, and endocrine and exocrine carcinomas (Li et al. 2014)
FRA17B	APH	17q23.1	0.071		HPV integration site in cervical tumor CC226 (Thorland et al. 2000)
FRA18A	APH	18q12.2	0.475		High frequency of LOH on chr 18 in esophageal squamous cell carcinoma (Karkera et al. 2000)
FRA18B	APH	18q21.3	0.182	<i>DCC</i>	Found as FSs with frequent interstitial deletions in HIV lymphomas (Capello et al. 2010)
FRA18C*	APH	18q22.1–18q22.2	0.067	<i>DOK6</i>	FS expression in the father of a patient with Beckwith-Wiedemann syndrome and a chromosome truncation 18q22-qter (Debacker et al. 2007)
FRA20B	APH	20p12.2			
FRA22B	APH	22q12.2	0.275		Elevated FS expression in bone marrow and peripheral blood of young cigarette smokers (Kao-Shan et al. 1987)
FRAXB*	APH	Xp22.31	5.494	<i>STS, GSI HDHD1 MIR4767</i>	Deletions within FRAXB seen in 15% of primary tumors and cell lines examined (Arft et al. 2002)
FRAXC	APH	Xq22.1	2.121	<i>DMD, IL1RAPL1</i>	
FRAXD	APH	Xq27.2	0.209		
FRA1H*	5-AZ	1q41–q42.1	0.129	<i>USH2A, ESRRG, MIRN194–1, MIRN215</i>	
FRA1J	5-AZ	1q12	0.12		
FRA9F	5-AZ	9q12	0.453		
FRA19A	5-AZ	19q13	0.053		
FRA4B	BrdU	4q12	0.142		
FRA5A	BrdU	5p13	0.191		
FRA5B	BrdU	5q15			
FRA6D	BrdU	6q13	0.031		
FRA9C	BrdU	9p21			
FRA10C	BrdU	10q21	0.089		
FRA13B	BrdU	13q21			
FRA4E	UC	4q27	0.382		

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Molecularly mapped fragile sites are indicated by an asterisk

5-AZ, 5-azacytidine, *APH* aphidicolin, *BrdU* bromodeoxyuridine, *UC* unclassified, *FS* fragile site, *LOH* loss of heterozygosity, *MS*/microsatellite instability, *ASD* autism spectrum disorder

^aData derived from Mrasek et al. (2010)

Table 21.3

Distribution of *RLFSs* in the genes located in 1973 *RFS*-associated and 13 cloned *CFS*-associated genes vs. all annotated genes in the genome

No. of <i>RLFSs</i> per gene	<i>RFSs</i>		<i>CFSs</i>		Whole genome	
	No. of genes	%	No. of genes	%	No. of genes	%
1	416	21.08	0		16,362	41.19
2	279	14.14	1	7.69	7,910	19.91
3	219	11.10	2	15.38	4,563	11.49
4	164	8.31	1	7.69	2,880	7.25
5	137	6.94	0		1,992	5.02
6–10	366	18.55	2	15.38	3,811	9.59
11–50	362	18.35	7	53.85	2,132	5.37
51–100	24	1.22	0		59	0.15
> 100	6	0.31	0		11	0.03
Total	1973	100	13	100	39,720	100

Statistics from the whole genome were previously reported (Wongsurawat et al. 2012)