

WWOX, large common fragile site genes, and cancer

Ge Gao and David I Smith

Division of Experimental Pathology, Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN 55905, USA
Corresponding author: David I Smith. Email: smith.david@mayo.edu

Abstract

WWOX is a gene that spans an extremely large chromosomal region. It is derived from within chromosomal band 16q23.2 which is a region with frequent deletions and other alterations in a variety of different cancers. This chromosomal band also contains the FRA16D common fragile site (CFS). CFSs are chromosomal regions found in all individuals which are highly unstable. WWOX has also been demonstrated to function as a tumor suppressor that is involved in the development of many cancers. Two other highly unstable CFSs, FRA3B (3p14.2) and FRA6E (6q26), also span extremely large genes, FHIT and PARK2, respectively, and these two genes are also found to be important tumor suppressors. There are a number of interesting similarities between these three large CFS genes. In spite of the fact that they are derived from some of the most unstable chromosomal regions in the genome, they are found to be highly evolutionarily conserved and the chromosomal region spanning the mouse homologs of both WWOX and FHIT are also CFSs in mice. Many of the other CFSs also span extremely large genes and many of these are very attractive tumor suppressor candidates. WWOX is therefore a member of a very interesting family of very large CFS genes.

Keywords: WWOX, common fragile sites, very large genes, cancer

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Introduction

Cancer development is a multistep complex process that is associated with different genetic alterations including the activation of oncogenes, inhibition of tumor suppressors, as well as numerous deletions and chromosomal rearrangements. It has been known that chromosome gaps and breaks which are often located in the common fragile sites (CFS) are involved in the early stages of malignant transformation. CFS are large chromosome regions that are prone to breakage upon replication stress. Although the mechanism responsible for CFS instability has remained elusive, the CFS deletions have been recognized as the most frequent genetic alterations observed during the development of many different cancers.

WWOX, located in the chromosome region 16q23.3-24.1, spans the second most active CFS, FRA16D. Homozygous deletions and loss of heterozygosity (LOH) in the chromosome 16q23 region as well as reduced mRNA and protein expression of WWOX have been observed in many human malignancies including breast, prostate, ovary, lung, and stomach cancers. Restoration of WWOX expression in human cancer cell lines and targeted deletion of *Wwox* in animal models have demonstrated its tumor suppressor function. In this review, we will discuss the essential role of WWOX in cancer development including its involvement in different signaling pathways in carcinogenesis as well as

its role in genome stability as a large CFS gene. We will also discuss the entire family of very large CFS genes that may also play important roles in cancer development.

WWOX is a very large gene spanned by the FRA16D CFS

Chromosomal band 16q23.2 is a hot spot for deletions and other alterations in a variety of different cancers including breast, ovarian and prostate cancers.¹ Translocations within this chromosome band t(14q32;16q23) are also found in about 25% of multiple myelomas.² A search for genes derived within this region that could be important targets of these deletions led to the discovery of WWOX (WW domain containing oxidoreductase). WWOX is an extremely large gene which spans over 1.1 megabases (Mb) within 16q23.2.^{3,4} There are actually 40 human genes that span over 1 Mb of genomic DNA, and WWOX is the 33rd largest of these.⁵ Despite the fact that this gene spans such a large genomic region, it is comprised of only nine small exons and has a final processed transcript that is relatively small (2.1 Kb). The amino acid sequence encoded by this gene revealed that it contains two WW domains and an oxidoreductase at its carboxy terminal end.³

This chromosomal band also contains the FRA16D CFS which is a region of profound genomic instability. CFSs are hot spots for deletions and other alterations in a variety of

different cancers. There have been 90 CFS regions described within the human genome. They are large regions of genomic instability found in all individuals when cells are cultured in the presence of DNA replication inhibitors such as aphidicolin or under stress conditions.^{6,7} FRA16D is the second most frequently expressed CFS in lymphocytes in the human genome. The assay to characterize a CFS region is a cumbersome cytogenetic-based assay where large insert clones (such as bacterial artificial chromosomes) are used as fluorescent probes against metaphase chromosomes cultured in the presence of aphidicolin. Individual BAC clones are found to hybridize proximal to the region of decondensation/breakage in some metaphases, distal in others and actually crossing in a few. Hence, CFS regions are considerably larger than the 200 Kb BAC clones used to define them. Utilizing a series of large insert clones derived from within chromosomal band 16q23.2, we previously demonstrated that the full size of the FRA16D region of instability is just over 2 megabases (Mb) in size. Unlike the rare fragile sites, the FRA16D CFS did not contain any triplet, mini- or microsatellite repeats, thus the mechanism of its instability appears to be distinct from those of the rare fragile sites. *WWOX* is contained entirely within this highly unstable region. Also contained within this region are all the 16q23.2 deletions in various cancers and the translocations observed in multiple myelomas.^{8,9}

WWOX and FRA16D is highly conserved in mice

CFSs are not only found in humans, but have been reported to be found in different mammalian species including mouse, cow, cat, and dog. Thus, investigation of the conserved sequences between species could be useful in studying the CFSs and the genes contained within these large regions of instability. This could also give insights into the mechanism for the instability within these large regions. To determine if the FRA16D/*WWOX* is conserved between species, our group searched for the homolog of the human *WWOX* gene, and identified that the mouse *Wwox* gene had high sequence similarity to the human *WWOX*. The cDNA and protein alignments demonstrated that the two orthologs are almost identical in exon regions but, in contrast to humans, there were relatively small introns in mice. The intron exon boundaries and the genomic structure were also determined and the overall genomic organization of *Wwox* is almost identical to the human *WWOX* on 16q23 although somewhat smaller. Overall, the mouse and human genomic sequences are similar, with 32.4% repeats in human and 41.4% in mouse. The GC content is also very similar, with 42.3% in human and 45.32% in mouse. Not only was there similarity between the *WWOX* and *Wwox* genes, the region surrounding *Wwox* is found to be a CFS (Fra8E1) in mice. The contig of BAC and YAC clones across the mouse genome containing the *Wwox1* and surrounding sequences determined that the mouse Fra8E1 had high similarity to human FRA16D.¹⁰

The aberrant expression of WWOX in different tumors and its clinical implications

Alterations within the large *WWOX* gene such as loss of heterozygosity (LOH), deletions within this very large gene, and increased promoter methylation have been observed in many different cancer types. Among all those, the most well and extensively studied cancers are those of the breast and lung.

WWOX was first identified as a gene involved in cancer development because the chromosome region 16q23.3-24.1 is an area that was commonly affected by allelic losses in breast cancer. Mutation screening of *WWOX* exons from a panel of breast cancer cell lines which are mostly hemizygous for the 16q genomic region indicated that there were no point mutations observed within the coding region of this gene. However, homozygous deletions and translocation break points have been mapped to the intronic regions of *WWOX*, all of which suggested that it might function as a tumor suppressor.³ A comparison of *WWOX* expression between normal breast tissues and breast cancer cell lines and primary tumors revealed that there was frequently reduced expression of *WWOX* RNA transcripts in many breast cancers. In addition, alternative *WWOX* transcripts lacking exons 6, 7, and 8 were detected in tumor tissues suggesting that it could play a role in breast cancer progression.^{11,12} Absent or reduced *WWOX* protein expression was also observed in invasive breast cancer tissues when compared with normal breast epithelium. It is very interesting to note that mRNA expression by Northern blot analysis revealed that the highest normal expression of *WWOX* was observed in hormonally regulated tissues such as testis, ovary, and prostate. This expression pattern and the presence of a short-chain dehydrogenase/reductase domain within the encoded protein suggest a role for *WWOX* in steroid metabolism.³ There was more significant *WWOX* loss in ER-responsive tissues and a strong correlation between *WWOX* expression and estrogen receptor status.¹³ An analysis of the correlation between decreased expression of *WWOX* and clinical outcome in breast cancer patients revealed that reduced *WWOX* expression was associated with bad prognosis. In contrast, tumors that did not have any change in *WWOX* expression or those that actually had increased *WWOX* expression were associated with improved disease-free survival.^{14,15}

An analysis of *WWOX* in non-small cell lung cancer (NSCLC) revealed *WWOX* transcripts with missing exons as well as LOH in primary tumors and cell lines.¹⁶ In a xenograft mouse model in which primary lung cancer cells were transfected with the mouse *Wwox* gene, the tumor size and weight were significantly lower than in mice that were transfected with a blank plasmid. In addition, the apoptosis level of primary lung cancer cell lines transfected with the *WWOX* gene was significantly higher than that observed in the blank plasmid transfected and non-transfected lung cancer cells.¹⁷ Studies also showed that conditional expression of wild type *WWOX*, but not mutant *WWOX* could suppress the clonogenic survival of non-small cell lung cancer cell lines as well as tumor growth *in vitro*. Preserved intratumoral *WWOX* expression was also

found to be associated with improved outcome in NSCLC patients and could serve as a prognostic biomarker in surgically resected, early staged NSCLC.¹⁸

Alterations in *WWOX* have also been observed in a variety of other cancers. Decreased expression of *WWOX*, transcripts missing exons, gene deletions and loss or decreased protein expression was also found in human osteosarcomas, hepatocellular carcinoma cell lines, gastric adenocarcinoma primary cancers, pancreatic primary tumors, pancreatic cancer cell lines and esophageal squamous cell carcinomas.^{19–23} Recent studies in NPC (nasopharyngeal carcinoma) revealed that the expression of *WWOX* in tumor tissues was significantly downregulated compared with that in non-tumorous tissues and decreased expression of *WWOX* was significantly correlated with clinical TNM stages. Methylation of *WWOX* was detected in most *WWOX* protein negative tissues suggesting that methylation of the *WWOX* promoter may regulate its expression.²⁴ Moreover, *Wwox* hypomorphic mice display a higher incidence of B-cell lymphomas and develop testicular atrophy.²⁵ The association between *WWOX* and disease prognosis was also found as deletion of 16q was associated with a worse clinical overall survival in multiple myeloma.²⁶

WWOX tumor suppressor's function

The decreased expression and the absence of *WWOX* observed in so many different cancers suggested that loss of *WWOX* could result in a growth advantage in transformed cells. Different *in vitro* and *in vivo* functional studies have indicated *WWOX*'s role as a tumor suppressor in both cell lines and animal models. It has been shown that restoration of *WWOX* in various cancer-derived cell lines that had no or low expression of *WWOX* could result in cell growth inhibition. In addition, ectopic expression of *WWOX* in cancer cells could also inhibit cell growth and cell tumorigenicity in both breast and lung cancer cell lines.^{27,28} *Wwox* knock-out in mice resulted in mice prone to develop osteosarcomas and *Wwox*-heterozygous mice had a higher incidence of spontaneous lung and mammary tumors as compared to their wild-type matched littermates.²⁹ Additional studies also indicated that the inactivation of the *Wwox* gene led to enhanced esophageal/forestomach tumorigenesis induced by N-nitrosomethylbenzylamine in *Wwox*+/- heterozygotes mice as compared to *Wwox*+/+ wild type mice.³⁰

There might be different mechanisms involved in *WWOX*'s tumor suppressor function. The WW domain, a component of *WWOX*, is a protein module mediating protein interactions that are often found in many structural and signaling proteins involved in different signaling processes. The WW domain is named after the presence of the two conserved tryptophans (W) residues and other proteins containing WW domains are known to play critical roles in tumorigenesis. For example, YAP, another gene encoding a protein with a WW domain, located at chromosome 11q22, is often found to have elevated protein levels in multiple cancers including those of the breast, liver, lung, colon, and ovary.^{31–34} This protein is involved in the Hippo pathway

and inactivation of YAP could cause cell contact inhibition and thus control tissue growth.³⁵ ITCH, an E3 ubiquitin ligase, can form complexes with the large tumor suppressor 1 (LATS1) through its WW domain and it works as a negative regulator of LATS1 promoting its degradation, thus playing an important role in tumorigenesis.³⁶ Similarly, *WWOX*, via its WW domain, could bind to PPxY-containing proteins and thus plays multifunctional roles in inhibiting tumorigenesis which include suppressing their oncogenic transcriptional activities, inhibiting apoptosis and decreasing migration. The interaction between *WWOX* and the c-Jun, ErbB-4 could inhibit their transcriptional activity and thus inhibit their proto-oncogenes activity.^{37,38} It was also shown that forced expression of *WWOX* could decrease FGF2-mediated proliferation and enhanced JNK inhibitor-induced apoptosis in human hepatocellular carcinoma cells.³⁹ Recent studies using human lung adenocarcinoma cell lines revealed that ectopic expression of *WWOX* could cause activation of procaspase-3 and caspase-9, and the release of cytochrome C thus leads to apoptosis.⁴⁰ p73, the p53 homolog, was also identified as the binding partner of *WWOX* through its PPxY motif in the C-terminal domain. Tyrosin (Y) 33 in the first domain of *WWOX* was revealed as the target for phosphorylation by the Src kinase family and Src kinase mediated the phosphorylation of *WWOX* which enhances its interaction with p73. It was hypothesized that the interaction between p73 and *WWOX* could result in enhancing transcriptional independent apoptosis.⁴¹ The mechanism whereby *WWOX* acts as a tumor suppressor also involves the modulation of the interaction between tumor cells and the extracellular matrix which thus inhibits tumor metastasis.⁴² It was reported that stable transfection of *WWOX* into human PEO1 ovarian cancer cells which contains a homozygous *WWOX* deletion could reduce the decreased attachment and migration on fibronectin, which is the extracellular matrix component important for metastasis. A recent characterization of *Wwox* inactivation in murine mammary gland development also demonstrated that reduction of *Wwox* expression is associated with increased levels of fibronectin which is also a component of the extracellular matrix, thus leading to impaired mammary ductal growth. *In vitro* experiments also showed that shRNA knockdown of *WWOX* could increase fibronectin and enhance cell survival and impaired growth in three-dimensional culture matrigel assay in MCF10A breast cell lines.⁴³ In addition, *WWOX* also plays a role in DNA damage response. Multiple reports have indicated altered *WWOX* expression after exposure to certain carcinogens. For example, decreased *Wwox* mRNA and protein level were observed in murine embryonic fibroblasts (MEF) after exposure to UV; altered *WWOX* expression observed in gastric carcinoma is known to be strongly associated with dietary carcinogens, and the *WWOX* alterations observed in esophageal and lung carcinomas were also related to tobacco and alcohol use. In addition, Abu-Odeh *et al.*⁴⁴ also reported that *Wwox* deficiency could result in reduced activation of the ataxia telangiectasia-mutated (ATM) checkpoint kinase, inefficient induction and maintenance of γ -H2AX foci, and impaired DNA repair, indicating its direct role in DNA damage response and DNA repair.

Functionally, *WWOX* is not only a tumor suppressor, it also participates in metabolic reactions and cell death signaling during neuron development. The *Wwox* deficient mice displayed metabolic disorders with impaired serum level of lipids and abnormal level of electrolytes, along with growth retardation and impaired steroidogenic enzyme levels.^{45,46} *WWOX* was also found to be involved in dopaminergic neurotoxin MPP⁺ (1-methyl-4-phenyl-pyridinium)-induced neurodegeneration. It has been shown that *WWOX* protein was upregulated and phosphorylated at Tyr33 (or activated) in injured neurons in the striatum and cortex in MPP⁺ treated rat brains. However, dephosphorylation of Tyr33 in *WWOX* could abolish this neurodegeneration which indicates that activated *WWOX* plays an essential role in MPP⁺-induced neuronal death.⁴⁷ Aqeilan group also indicated *WWOX*'s emerging role in regulation of aerobic glycolysis. They identified that *WWOX* could interact with HIF1 α via its first WW domain and modulate its transactivation function. *Wwox*-deficient cells exhibited increased HIF1 α levels and displayed increased glucose uptake, and these cells also displayed increased GLUT1 level *in vivo* and are more tumorigenic. They also found that *WWOX* expression was inversely correlated with GLUT1 in breast cancer samples indicating *WWOX*'s role in cancer metabolism.⁴⁸

CFSs and large genes

Thus *WWOX* is an extremely large gene which is spanned by the highly unstable CFS FRA16D and it functions as an important tumor suppressor involved in the development of a variety of different cancers. Many of the other CFS regions are also hot spots for deletions and other alterations in different cancers. The three most frequently expressed of these regions are FRA3B (3p14.2), the FRA16D/*WWOX* (16q23.2) region and FRA6E (6q26).⁶ While *WWOX* was found to be a very large CFS gene, it was not the first large CFS gene identified. Indeed, the first very large gene which was found to be spanned by a highly unstable CFS region was *FHIT* within FRA3B.

FRA3B and FHIT

The most frequently expressed of all CFSs is FRA3B which resides within chromosomal band 3p14.2.⁴⁹ Similar to chromosomal band 16q23.2 and FRA16D, the 3p14.2 region is frequently deleted or altered in a variety of different cancers. In addition, it was found that individuals from a family who had a balanced reciprocal translocation t(3;8)(3p14.2;8q24.23) involving this chromosomal region had a very high probability of developing renal cell carcinoma.⁵⁰ In cervical cancer, this region was often found to be the site of human papillomavirus (HPV) integrations.⁵¹ All these suggested that there might be a tumor suppressor gene that played a role in cancer development within this region.

The FRA3B CFS was eventually localized when large insert yeast artificial chromosomes derived from this region were utilized as FISH-based probes against aphidicolin-induced metaphases from lymphocytes. A 1.1

megabase YAC clone was identified which hybridized sometimes proximal to the region of decondensation/breakage in some metaphases, distal in others and even crossing this region in a few metaphases.⁵² Thus, the entire FRA3B region encompasses a region larger than this YAC clone. This YAC clone also contained the hereditary renal cell carcinoma translocation breakpoint and the HPV integrations which occurred within 3p14.2.^{52,53}

A search for genes within this region led to the identification of another extremely large gene *FHIT* (fragile histidine triad), which encoded a protein with a histidine triad motif. Similar to *WWOX*, in spite of the fact that the genomic region spanned by this gene was 1.5 Mb, it only contains 10 small exons and the full size of the final processed transcript was only 1.1 Kb.⁵⁴ Deletions, loss of expression and other alterations of *FHIT* were frequently observed in a variety of different cancers including breast, lung, cervical cancers and, B-cell lymphoma.^{55–58} *FHIT* was also demonstrated to function as a tumor suppressor as re-introduction of *FHIT* into cancer-derived cell lines which had either deletions in this region or greatly reduced expression of the *FHIT* protein resulted in growth inhibition and the induction of apoptosis.^{59,60} When *FHIT* knock-out mice were made, they were found to be tumor-prone and re-introduction of a functional *FHIT* gene into these mice suppressed tumor formation.⁶¹

Recent reports also indicated that *Fhit*-deficient cells exhibit spontaneous DNA breaks indicating its emerging role as the guardian of the genome. Both the loss of *Fhit* expression and genomic instability were detected and observed in many precancerous lesions. The Huebner group has found that the loss of *Fhit* protein could cause reduced expression of thymidine kinase, dTTP imbalance, impaired DNA replication fork progression and spontaneous DNA breaks which all lead to increased genome instability.⁶² They concluded that *FHIT* loss is among the earliest changes in the preneoplastic process which initiates the onset of genome instability leading to tumorigenesis. In addition, Hosseini *et al.* also examined the markers of genome instability in epithelial cells with *FHIT* deficiency and found those cells exhibited increases in fragile breaks in both γ H2AX and 53BP1 foci in G1 phase. These findings also support that FRA3B/*FHIT* is a caretaker gene which plays important roles in the maintenance of genome stability.⁶³

FHIT provided an important link between chromosomal instability and cancer as this gene was highly susceptible to alterations within the FRA3B region and many pre-cancerous lesions, especially those of lung cancer had inactivation of *FHIT*. In addition, the carcinogens in cigarette smoke could induce alterations in FRA3B that resulted in decreased *FHIT* expression.⁶⁴

The similarity between FRA16D/*WWOX* and FRA3B/*FHIT*

There are thus a number of key similarities between the FRA16D region and *WWOX* and the FRA3B region and *FHIT*. *FHIT* and *WWOX* span very large genomic regions but with final processed transcripts that are really small.

Despite that fact the *FHIT* and *WWOX* reside within two of the most unstable genomic regions, there is high sequence conservation in species of both *FHIT*/FRA3B and *WWOX*/FRA16D. The *Fhit* and the *Wwox* genes in mice have the same overall organization as the human *FHIT* and *WWOX* genes and the genomic regions surrounding these genes are also highly unstable CFSs in mice. The human FRA3B and FRA16D CFSs and the mouse Fra14A2 and Fra8E1 are both associated with recurring translocations. Furthermore, mice carrying one or two inactivated alleles (*Fhit*+/-, *Wwox*+/- or *Fhit*-/-, *Wwox*-/-) developed spontaneous tumors and showed an increased incidence of tumor development. Moreover, the decrease or loss of the expression of both genes was reported in many different cancers but with rare point mutations.

Despite the similarity between the FRA3B/*FHIT* and FRA16D/*WWOX*, the initial study in analyzing the distribution of aphidicolin-induced CFSs in human embryonic cells with different origins revealed that the instability of the fragile sites was differentially expressed in different cell types. In particular, fragility within FRA16D was often found in both lymphocytes and fibroblasts, while instability within FRA3B was much less frequently observed in fibroblasts than lymphoblastoid cells.⁶⁵⁻⁶⁷ Thus, it is not a surprise to find the coordinate loss of both FRA3B/*FHIT* and FRA16D/*WWOX* in certain hematopoietic malignancies. The studies in both human primary hematopoietic neoplasias and a series of leukemia cell lines revealed that each gene showed aberrations or absence of expression in many primary cancers and cell lines, and the occurrence of aberrant *FHIT* correlated significantly with the occurrence of *WWOX* alterations.⁶⁸ The coordinate loss of expression of these two genes was also found in 85% of primary effusion lymphoma (PEL) cell lines.

The recent CFS profiling from Le Tallec *et al.* and Hosseini *et al.* in a variety of different cell lines revealed that epithelial cells display different fragile sites as compared to primary lymphocytes and fibroblasts, and that cell lines of the same cell type tend to share more CFSs than cell lines with different cell types, thus CFS distribution is cell type dependent.⁶⁹ The study from Hosseini *et al.* also indicated that epithelial cells exhibited various hierarchies of the fragile sites; some more active epithelial cell fragile sites are not necessarily those that are most often altered in epithelial cancer cells and those fragile sites that are often deleted in epithelial cancers are not necessarily among the most active fragile site list which was derived from examining fragility in lymphocytes.⁷⁰

The evaluation of both *FHIT* and *WWOX* in one specific cancer also has significance in elucidating potential mechanisms involved in cancer development. For example, when both *FHIT* and *WWOX* and other tumor suppressors were evaluated in pancreaticobiliary cancers, it was found that *FHIT* and *WWOX* were ubiquitously expressed in benign samples but had significantly coordinately reduced expression in pancreatic, gallbladder and ampullary cancers, while neither *FHIT* nor *WWOX* expression correlated with expression of other tumor suppressors.⁷¹ This suggested that both *FHIT* and *WWOX* possibly share the same mechanisms as tumor suppressors that are somehow

related to their being located within highly unstable CFS regions. Messenger RNA expression of both *FHIT* and *WWOX* were also reported significantly lower in nasopharyngeal carcinoma patients and this loss is associated with poor histologic differentiation and advanced clinical stages.^{72,73}

FRA6E and *PARK2*

Chromosomal band 6q26 is another chromosomal band which is a hot-spot for deletions and other alterations in multiple cancers. This region was found to have a high frequency of LOH in squamous cell lung, ovarian, hepatocellular and breast cancers.⁷⁴⁻⁷⁷ By using FISH-based probes with large insert BAC clones derived from the 6q26 band – the strategy also used for localizing FRA16D and FRA3B, we were able to define FRA6E. Utilizing seven BAC clones across this region,⁷⁸ we found that FRA6E spans approximately 3.6 Mb and contained within this unstable region was another extremely large gene *PARK2*. Similar to *WWOX* and *FHIT*, *PARK2* spans 1.36 Mb but is comprised of 11 small exons with a final processed transcript of only 2.3 Kb.⁷⁹ *PARK2* was first identified as a mutational target in patients with autosomal recessive juvenile Parkinsonism (ARJP) and the protein is known to contain an ubiquitin-like domain at its N-terminus and two RING finger motifs and an IBR at its C terminus. It encoded an E3 ubiquitin-protein ligase which binds to E2 ubiquitin-conjugating enzymes.⁸⁰

Reduced or absent *PARK2* transcripts have been observed in a variety of different cancers including ovarian, breast, renal, lung, and sporadic colorectal cancer and this frequent loss suggested that the genomic deletions observed across this gene might lead to tumor initiation and development.⁸¹⁻⁸⁵ It has been shown that germline *PARK2* mutations could cause neural dysfunction, and somatic *PARK2* mutations could decrease *PARK2*'s E3 ligase activity, compromising its ability to ubiquitinate cyclin E thus resulting in mitotic instability, which suggests that it could also have a tumor suppressor function.⁸⁶ Recently, it also has been shown that the *PARK2* E3 ubiquitin ligase is an important coordinator of G1/S-phase cyclin turnover. It was discovered that *PARK2* is a component of two novel CRL complexes named PCF4 (containing FBX4) and PCF7 (containing FBXW7) that could target cyclin D1 and cyclin E1, respectively, for degradation.⁸⁷

An entire family of large CFS genes

As all three of the most unstable CFS regions spanned very large genes and all three of these genes were demonstrated to function as tumor suppressors, it was interesting to know whether other CFS regions also spanned large genes and if other large CFS genes played roles in cancer development. In 2005, we obtained a list of the largest human genes and found that there are 40 genes which spanned over 1 Mb and 200 genes whose genomic region spans over 500 Kb. Table 1 contains all 40 genes which span greater than 1 Mb of genomic DNA. Also contained on this table are the size of each gene, their chromosomal location, and the number of exons,

Table 1 The 40 human genes that span greater than 1 Mb of genomic sequence

	Gene name	Chromosome	Size	Exons/FPT
1	<i>CNTNAP2</i>	7q35	2304258	25/8107
2	<i>DMD</i>	Xp21.1	2092287	79/13957
3	<i>CSMD1</i>	8p23.2	2056709	70/11580
4	<i>LRP1B</i>	2q22.1	1900275	91/16556
5	<i>CTNNA3</i>	10q21.3	1775996	18/3024
6	<i>NRXN3</i>	14q24.3	1691449	21/6356
7	<i>A2BP</i>	16p13.2	1691217	16/2279
8	<i>DAB-1</i>	1p32.3	1548827	21/2683
9	<i>PDE4D</i>	5q11.2	1513407	17/2465
10	<i>FHIT</i>	3p14.2	1499181	9/1095
11	<i>KIAA1680</i>	4q22.1	1474315	11/5833
12	<i>GPC5</i>	13q31.3	1468199	8/2588
13	<i>GRID2</i>	4q22.3	1467842	16/3024
14	<i>DLG2</i>	11q14.1	1463760	23/3071
15	<i>AIP1</i>	7q21.11	1436474	21/6795
16	<i>DPP10</i>	2q14.1	1402038	26/4905
17	<i>PARK2</i>	6q26	1379130	12/2960
18	<i>ILIRAPL1</i>	Xp21.2	1368379	11/2711
19	<i>PRKG1</i>	10q21.1	1302704	18/2213
20	<i>EB-1</i>	12q23.1	1248678	26/3750
21	<i>CSMD3</i>	8q23.2	1213952	69/12486
22	<i>IL1RAPL2</i>	Xq22.3	1200827	11/2985
23	<i>AUTS2</i>	7q11.22	1193536	19/5972
24	<i>DCC</i>	18q21.1	1190131	29/4608
25	<i>GPC6</i>	13q31.3	1176822	9/2731
26	<i>CDH13</i>	16q23.2	1169565	15/3926
27	<i>ERBB4</i>	2q34	1156473	28/5484
28	<i>ACCN1</i>	17q11.2	1143718	10/2748
29	<i>CTNNA2</i>	2p12	1135782	18/3853
30	<i>WD repeat</i>	2q24	1126043	16/2132
31	<i>DKFZp686H</i>	11q25	1117478	8/6830
32	<i>PTPRT</i>	20q12	1117144	32/12680
33	<i>WWOX</i>	16q23.2	1113013	9/2264
34	<i>NRXN1</i>	2p16.3	1109951	21/8114
35	<i>IGSF4D</i>	3p12.1	1109105	10/3315
36	<i>CDH12</i>	5p14.3	1102578	15/4167
37	<i>PAR3L</i>	2q33.3	1069815	23/4176
38	<i>PTPRN2</i>	7q36.3	1048712	22/4735
39	<i>SOX5</i>	12p12.1	1030095	18/4492
40	<i>TCBA1</i>	6q22.31	1021499	8/3183

and the size of each gene's final processed transcript. Many of these very large genes are derived from chromosomal bands containing a CFS. To prove if these genes are actually located within the closely associated CFSs, we took BAC clones which spanned a portion of these genes (in some instances, two BACs were obtained with one derived from the 5' end of a gene and the other derived from the 3' end) and used them as FISH-based probes against metaphase preparations of lymphocytes that had been exposed to 0.4 μ M aphidicolin for 24 h.

The FISH-based analysis of FRA16D, FRA3B, and FRA6E was relatively easy as each of these CFS regions are highly

unstable and most metaphase spreads had breakage/decondensation within each of these regions, while other CFS regions sometimes only had breakage/decondensation within relatively few metaphases. Thus, for some of the CFSs, it was necessary to search many hundreds of metaphases from cells cultured in the presence of aphidicolin searching for decondensation/breakage within each respective CFS region. To definitively determine if a specific BAC (and thus the large gene spanning that BAC) was within a CFS region, it was necessary to find that BAC hybridizing proximal to the region of decondensation/breakage in one metaphase and distal in another. We carried out this type of analysis with a number of the very large genes including the largest known human gene, *CNTNAP2* (derived from within chromosomal band 7q35), the second largest known gene *DMD* (derived from within chromosomal band Xp21.1), *CTNNA3* (derived from within chromosomal band 10q21.3), *DAB1* (derived from within chromosomal band 1p32.3), *DLG2* (derived from within chromosomal band 11q14.1), and *RORA* (derived from within chromosomal band 15q22.2). All of these large genes were found to be contained within their respective CFS regions.

Other groups identified other very large genes that were also localized within a CFS. For example, Rozier *et al.* identified that *GRID2* localized within FRA4G and *GRID2* mutations in mice could produce the neurological mutant Lurcher.⁸⁸ They also identified the *GRID* mouse homolog located within mouse CFS Fra6C1. Similar to *WWOX* and *FHIT*, there is considerable conservation between the region surrounding *GRID2* in humans and mice. This was the first association between an inherited disease and cancer, as deletions and other alterations within *GRID2* are observed in multiple cancers. Similarly, the large neurobeachin gene (*NBEA*) was identified localized within the FRA13A CFS by Savelyeva *et al.*⁸⁹ To date, there have been 26 very large genes found localized within CFS regions (Table 2). Table 2 shows the genes, the chromosome location, the size of those genes, and the CFS that spans each of these genes.

It is clear that some very large genes are comprised of a very large number of exons (like *DMD* and *LRP1B*) with relatively large final processed transcripts, while others (like *WWOX*, *FHIT* and *PARK2*) have fewer exons and much smaller final processed transcripts. Hence, there is no correlation between the size of a genomic region that a gene spans and the size of its final processed transcript, and this is also true for genes that do not span such large genomic regions.

The reduced expression of many of these large CFS genes was also found in different cancers and some of these CFS large genes are very attractive tumor suppressors. For example, *RORA* was found to be frequently inactivated in breast, prostate and ovarian cancers while it was expressed in their normal tissues⁹⁰⁻⁹³; *DMD* and *IL1RAPL1* were dramatically underexpressed in every brain tumor cell line tested as compared to matched normal brain tissue, and it has also been demonstrated that mice with double mutants for dystrophin and dysferin were found to be predisposed to develop rhabdomyosarcoma^{94,95}; the deletion of FRA4G

Table 2 The 26 known large CFS genes and their CFS regions and size

	Gene symbol	Chromosome	CFS region	Size (bp)
1	CNTNAP2	7q35	FRA7I	2304258
2	DMD	Xp21.1	FRAXC	2092287
3	LRP1B	2q22.1	FRA2F	1900275
4	CTNNA3	10q22.2	FRA10D	1775996
5	DAB1	1p32.3	FRA1B	1548827
6	FHIT	3p14.2	FRA3B	1499181
7	CCSER1 (KIAA1680)	4q22.1	FRA4G	795996
8	GRID2	4q22.1	FRA4G	1467842
9	DLG2	11q14.1	FRA11F	1463760
10	PARK2	6q26	FRA6E	1379130
11	IL1RAPL1	Xq21.2	FRAXC	1368379
12	WWOX	16q23.2	FRA16D	1113013
13	SDK1	7p22.2	FRA7B	967552
14	PDGFRA	4q12	FRA4	920550
15	IMMP2L	7q31.1	FRA7K	899938
16	RORA	15q22.2	FRA15A	732040
17	PTPRC	1q31-32	FRA1K	118042
18	NBEA	13q13	FRA13A	730451
19	LARGE	22q12.3	FRA22B	647335
20	ARHGAP15	2q22.2-q22.3	FRA2F	639023
21	CTNNA1	5q31.2	FRA5B	181617
22	THSD7A	7p21.3	FRA7B	457654
23	MAD1L1	7q22	FRA7B	417157
24	DPYD	1p22	FRA1D	1096311
25	ESRRG	1q41	FRA1H	824826
26	USH2A	1q41	FRA1H	800503

which contains *GRID2* was also found in colon cancer; and the deletion of *FRA13A/NBEA* was also found in multiple myeloma.⁹⁶ Another very promising tumor suppressor candidate is the very large gene contained within *FRA2F* (2q22.1), *LRP1B*. This 1.9Mb very large gene has deletions in high-grade serous ovarian cancers and this is associated with acquired chemotherapy resistance to doxorubicin.⁹⁷

As most of the effort in studying the CFS large genes and their relationship to cancer has been focused specifically on the two most well-known large CFS genes *FHIT* and *WWOX*, the systematic profiling or analysis of all the known large CFS genes is limited in cancer studies. However, in addition to *FHIT* and *WWOX*, the concordant inactivation of two or more CFS large genes has been reported in different cancers. For example, it has also been shown that there were novel chimeric genes *PVT1-NBEA* and *PVT1-WWOX* that frequently occurred in multiple myelomas, in the presence of abnormal expression of *NBEA* and *WWOX*.⁹⁸ Genome-wide DNA-profiling of HIV-related B cell lymphomas revealed that the three known CFS tumor suppressor genes *FHIT* (*FRA3B*), *WWOX* (*FRA16D*), and *PARK2* (*FRA6E*) were frequently concordantly inactivated in HIV-positive non-Hodgkin lymphomas.⁹⁹

Our laboratory has been studying Oropharyngeal Squamous Cell Carcinoma (OPSCC), a subtype of head

and neck cancer which has been observed to be increasing in frequency due to more frequent HPV infections.¹⁰⁰ We analyzed gene expression in OPSCC using next generation sequencing and RNAseq and this enabled us to do a systemic analysis of the expression of all known CFS large genes to compare RNA expression in OPSCC tumors to matched normal oropharyngeal tissue obtained from the same patients. This analysis revealed that there was a select group of CFS large genes that had decreased expression in the tumor samples when compared to the matched normal tissue, while some other CFS large genes showed either increased expression or no changes in their expression. Large CFS genes with decreased expression included the two most known tumor suppressors *FHIT* and *PARK2*, and four other CFS large genes *DMD*, *DLG2*, *NBEA*, and *CTNNA3*. Validation experiments using quantitative reverse transcription real-time PCR in a much larger number of OPSCCs revealed that this selected group of CFS large genes had decreased expression in more than half of the samples analyzed.

Not all large CFS genes had decreased expression, however. *WWOX* had increased expression in most OPSCC tumor samples examined. Several other CFS large genes were also observed as having increased expression in the tumor samples examined similar to *WWOX*. This is not the first report of *WWOX* having increased expression in some tumors examined. Previously, Watanabe *et al.* observed elevated *WWOX* protein levels in gastric and breast carcinoma as compared to noncancerous cells, arguing whether *WWOX* did actually function as a tumor suppressor. They suggested that more evidence might be needed to demonstrate that it was a tumor suppressor.¹⁰¹

Our observation that some CFS large genes have decreased expression while others do not cannot be simply interpreted as the result of random genome instability within CFS regions. If it was due to random genomic instability in the CFS regions during carcinogenesis, we would have expected to see losses in the expression of all CFS genes, or more losses in the expression of the large CFS genes derived from the most highly unstable CFS regions. *WWOX* is derived from the second most unstable CFS region, and yet its expression was actually increased in many OPSCCs. It is possible that there is a selection for alterations in specific regions due to the important large genes that reside within them, or this could be due to some chromosome regions that are more sensitive to specific carcinogens that are involved in certain type of cancers. Alternatively, altered expression could be related to specific organs or tissues and tissue-specific expression of these genes. For example, compared to cancers arising in non-smokers, *FHIT* has been shown to have more significant alterations or loss in smokers in both lung cancers and cervical cancers.¹⁰²⁻¹⁰⁴ In addition, Thavathiru *et al.* demonstrated that expression of both *WWOX/FRA16D* and *FHIT/FRA3B* is downregulated by exposure to the carcinogens, UV, and Benzo(a)pyrene diol epoxide (BPDE) but not when exposed to ionizing radiation.¹⁰⁵

Whether the differential expressed large CFS genes observed in OPSCC has any clinical significance needs further investigation. There have been reports indicating that

the concordant loss of expression of both FHIT (FRA3B) and WWOX (FRA16) blocks apoptosis in lymphocytes in patients with thyroid cancer.¹⁰⁶ Recently, Le Tallec *et al.* performed common fragile site profiling in epithelial and erythroid cells and they found that over 50% of recurrent cancer deletions originate in CFSs that are associated with genes over 300 Kb in size.⁶⁹ If the decreased expression of specific large CFS genes is associated with patients' outcome, monitoring the expression of large CFS genes could prove to be a powerful prognostic marker to stratify patients for better treatment options.

Finally, why are so many very large genes contained within the CFS regions? In addition, why are there so many potential important tumor suppressors that are very large CFS genes? One possible answer for the first question was suggested by Helmrich *et al.*¹⁰⁷ They found that collisions between replication and transcription complexes cause common fragile site instability at the longest human genes. This is an intriguing hypothesis, but not all very large genes reside within CFS regions and not all unstable CFS span very large genes. The second question is also very interesting as it provides an important linkage between genomic instability and cancer development. One possibility is that these large CFS genes act as genomic sensors due to their locations and that too much damage disrupts this system and ends up promoting tumorigenesis. Clearly, much more work needs to be done on the CFS regions and the very large genes contained within them.

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