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PROSPECTS

Molecular Parameters of Genome Instability: Roles of Fragile Genes at Common Fragile Sites

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Abstract Common chromosome fragile sites occur at specific sequences within mammalian genomes that exhibit apparent single-stranded regions in mitotic chromosomes on exposure of cells to replication stress. Recent progress in the characterization of sequences, and more precise mapping of common fragile sites in mammalian and yeast genomes, has led to the exact placement of large common fragile regions straddling the borders of chromosomal G and R bands, with early and late replicating genomic regions, respectively, and could lead to breakthroughs in understanding the function of these evolutionarily conserved but highly recombinogenic chromosome elements. Deficiency of genes involved in DNA damage checkpoint responses, such as *ATR, CHK1, HUS1* leads to increased frequency of fragile site instability. Some of these fragile sites, particularly *FRA3B*, encode genes that are themselves involved in the protection of cells from DNA damage through various mechanisms. Protection of mammalian genomes from accumulation of DNA damage in somatic cells is critical during development, puberty and during the reproductive lifespan, and occurs through mechanisms involving surveillance of the genome for damage, signals to the cell cycle machinery to stop cell cycle progression, signals to repair machinery to repair damage, signals to resume cycling or initiate apoptotic programs, depending on the extent of damage and repair. When genes involved in these processes are altered or deleted, cancer can occur. The tumor suppressor gene, *FHIT* at the *FRA3B* locus, and possibly other fragile genes, is a common target of damage and paradoxically encodes a protein with roles in protection from DNA damage. J. Cell. Biochem. 104: 1525–1533, 2008.

Key words: common fragile sites; DNA damage checkpoint; *FHIT*; DNA repair

Common human chromosome fragile sites (CFSs) are specific chromosome regions that, in metaphase chromosomes of normal white blood cells of all individuals, show apparent breaks or gaps if the cells have been cultured under conditions of mild replicative stress. There are >80 such sites in the human genome and at least some of them are conserved in other mammalian species [Heimreich et al., 2006]. A number of these CFSs have been fully sequenced and sequences scanned for clues to the basis of fragility. These sites are highly recombinogenic, serving as sites for chromosome translocations, deletions, amplifications, and exogenous DNA integration, in preneoplastic, neoplastic, and other clonally expanded cell populations

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[Arlt et al., 2006]. CFSs tend to be large genomic regions, on the order of a megabase, are usually AT-rich, and sometimes late replicating. Late replication can contribute to fragility by allowing progress into the G2 phase with single-stranded DNA regions still unreplicated [Schwartz et al., 2006; Freudenreich, 2007].

Shortly after CFSs were discovered in the early 1980s [Glover et al., 1984], it was observed that many of these chromosome regions appeared to be coincident with chromosome regions non-randomly altered in specific cancers and the hypothesis that fragile sites might harbor genes with relevance to cancer development was born. This hypothesis has driven numerous studies to isolate genes at fragile sites and to understand the basis of the striking recombinogenicity of CFSs. The genes at the two most active CFSs, i.e., those sites showing the most frequent gaps in normal lymphocytes, have been characterized and their gene products, Fhit and Wwox, extensively examined for roles in cancer development [Huebner and Croce, 2003; Iliopoulos et al., 2006]. We will focus on these two fragile sites and their gene products in an exploration of the questions raised by the existence of fragile sites, their conservation across species, and the roles of the genes encompassing them.

COMMON FRAGILE SITES, GENERATORS OF CHROMOSOME INSTABILITY

Because CFSs are highly conserved across species [Heimreich et al., 2006] in spite of their propensity toward sequence alterations [Matsuyama et al., 2003], speculation about the meaning of fragile sites has suggested a range of functions: perhaps they serve as weak points in the genome that have allowed the chromosome recombination associated with evolution and speciation [Ruiz-Herrera et al., 2006]; perhaps there is some meaning behind the fact that they encode some large genes that may have significance in stress responses or neurological diseases [Smith et al., 2006]; maybe they are doomsday genes, in which accumulated alterations confer selection for cancer growth, inevitably eliminating individuals who are past their reproductive period; or perhaps they do not have a structural function but simply represent the genome regions that are most susceptible to mild replication stress that can lead to cell death or to aberrant repair.

Results of Zlotorynski et al. [2003] suggested that a shared molecular basis, conferred by sequences with a potential to form secondary structures that can perturb replication, is the basis of the fragility of rare fragile sites harboring AT-rich minisatellite repeats and aphidicolin-induced common fragile sites.

The same group of investigators showed that, following gene therapy of children with severe combined immunodeficiency using murine leukemia virus (MLV)-derived vector, two patients developed leukemia due to an activating vector integration near the *LMO2* gene. The integrations were in *FRA11E*, a common fragile site known to be involved in chromosomal breakpoints in tumors [Bester et al., 2006].

To examine the dynamics of mammalian genome evolution, Ruiz-Herrera et al., [2006] analyzed the distribution of syntenic blocks, evolutionary breakpoint regions, and evolutionary breakpoints taken from public databases available for mouse, rat, cattle, dog, pig, cat, horse and chicken, and examined them for tandem repeats and coincidence with human fragile sites. The results confirmed previous studies that showed the presence of chromosomal regions in the human genome that have been repeatedly used, as illustrated by a high breakpoint accumulation in certain chromosomes and chromosomal bands; there was a striking correspondence between fragile site location, positions of evolutionary breakpoints, and distribution of tandem repeats throughout the human genome, providing evidence that certain chromosomal regions in the human genome have been repeatedly used in the evolutionary process.

Debatisse et al., [2006] have made the very interesting and important observation that common fragile sites map at the junction of chromosomal bands replicating at different times in S phase, indicating that specific replication programs take place at CFSs (see Fig. 1, for illustration). The results suggested that CFSs remain incompletely replicated up to late G2, even in cells that had unperturbed S phases [El Achkar et al., 2005]; and as suggested by the placement of *FHIT* and *WWOX* exons in Figure 1, this may partially explain the position of the most frequently deleted regions in these two tumor suppressor genes, since the frequently deleted regions may fall in the late-replicating R bands of the two fragile regions, as illustrated. The recent demonstration that the

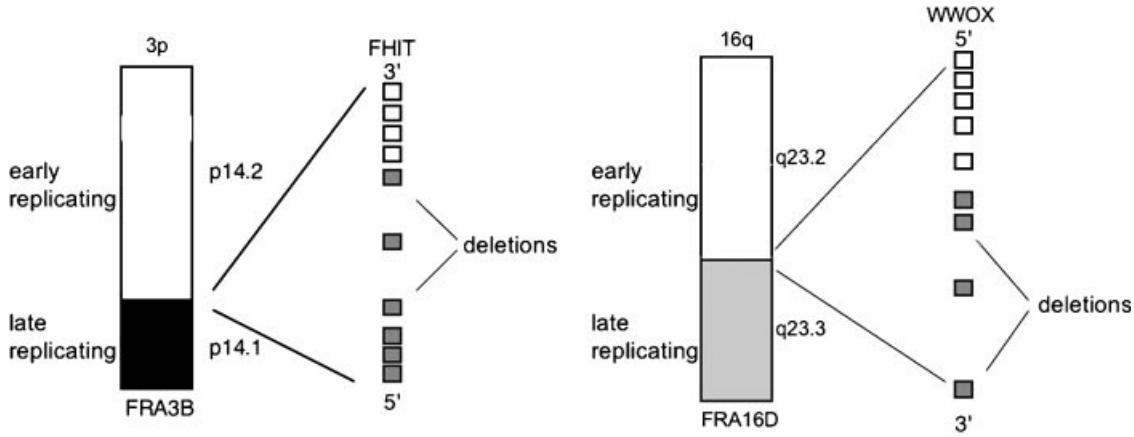

Fig. 1. Positions of *FHIT* and *WWOX* genes at the junction of late-replicating G dark bands and early replicating G light bands. Note that for each gene, the filled exons (gray filled boxes) may be in the portion of the gene that falls in the late-replicating band [based on the study of El Achkar et al., 2005]. These are also the regions of the two tumor suppressor genes exhibiting the highest frequency of genomic deletions.

function of Atr and its downstream targets are crucial to CFS stability may thus indicate that mitotic onset is delayed until the completion of their replication. It is now known that cells deficient in Atr, Chk1, Brca1, Hus1, Smc1, FancD2 [Arlt et al., 2006; Zhu and Weiss, 2007] are highly susceptible to the increased spontaneous occurrence of gaps/breaks at CFSs, so all of these gene products, involved in DNA damage responses, are important in the maintenance of the integrity of CFSs. The Debatisse group concludes that, together with the finding that the Atr DNA damage checkpoint pathway [Paulsen and Cimprich, 2007] is essential for the integrity of fragile sites, the results suggest that CFSs constitute integral "*cis*" components of the G2-M checkpoint.

GENOME INSTABILITY IN PRENEOPLASIAS

Precancerous cells undergo selective pressure to escape from the cell cycle block induced by checkpoint responses to DNA damage. Studies of hyperplasias and dysplasias [Bartkova et al., 2005; Gorgoulis et al., 2005] of skin and lung showed that from early dysplastic stages, an Atr/Atm-regulated DNA damage response network is activated, delaying or preventing cancer; allelic loss within the *FHIT* gene, at common fragile site *FRA3B*, is coincident with these events in early lesions. Mutations compromising this checkpoint, including defects in the Atr-Chk1 pathway, would allow cell proliferation, and tumor progression, leading to more DNA replication stress and increased genomic instability. Previous studies have shown involvement of repair associated proteins in control of fragile site integrity, including Atr, Brca1, Smc1, Fanconi anemia pathway proteins, Chk1 and Hus1 [Arlt et al., 2006; Zhu and Weiss, 2007]. A recent study showed that homologous recombination and non-homologous end-joining repair pathways regulate *FRA3B* fragile site stability, indicating that double-strand breaks are formed at common fragile sites due to replication perturbation [Schwartz et al., 2005]. The 1.7 MB *FHIT* gene encodes a 1.2 kb mRNA and a 16.8 kDa protein, and is frequently involved in biallelic loss and other chromosome abnormalities in tumors [Huebner and Croce, 2003; Iliopoulos et al., 2006]. Loss of Fhit expression is observed in premalignant lesions of esophagus, stomach, cervix, and other organs, suggesting that loss of Fhit expression, due to the susceptibility of *FHIT*/*FRA3B* to carcinogen damage, plays a role in initial stages of multistep carcinogenesis. There are even reports that increased frequency of common fragile site expression in normal white blood cells can predict predisposition to breast, ovarian, lung, and other cancers [Tunca et al., 2002; Dhillon et al., 2003], though these results are not widely accepted.

CONTRIBUTION OF THE FHIT PROTEIN TO GENOMIC INSTABILITY

Because fragile site expression in mitotic cells may result from unrepaired DNA damage, we

were curious very early about how the *FHIT* locus might be affected in cancers carrying mutations in familial cancer genes such as *BRCA1* and 2, that were known to be involved in DNA repair processes. Our first opportunity to examine such cancers came with a collection of Icelandic familial breast cancers carrying *BRCA2* mutations. We found a significantly higher frequency of LOH at the *FHIT* locus in the *BRCA2* mutant tumors compared to matched, sporadic breast cancers, possibly due to misrepaired double-strand breaks at *FRA3B* [Ingvarsson et al., 1999]. To determine if such genomic alterations led to Fhit inactivation, we also assessed the level of Fhit expression by immunohistochemical detection in sporadic tumors and cancers occurring in *BRCA2* 999del5 carriers. To determine if Fhit inactivation may have prognostic significance, we assessed expression of breast cancer markers and clinical features in sporadic tumors relative to Fhit expression. Of 40 consecutive sporadic breast carcinomas studied for tumor markers, 50% showed reduced Fhit expression. In these sporadic cancers, loss of Fhit expression was not correlated significantly with the presence or absence of other tumor markers. In the study of 58 sporadic and 34 *BRCA2* 999del5 Icelandic invasive cancers, there was a significant association of LOH at 3p14.2 with reduced expression of Fhit ($P = 0.001$); also the lower expression of Fhit and higher LOH at 3p14.2 in *BRCA2* 999del5 tumors relative to sporadic cancers was significant ($P = 0.002$). Thus, genetic alteration at the fragile site within the *FHIT* gene leads to the loss of Fhit protein in a significant fraction of sporadic breast cancers and a much larger fraction of familial breast cancers with an inherited *BRCA2* mutation, consistent with the idea that loss of *BRCA2* function affects the stability of the *FHIT*/*FRA3B* locus. Similarly, 22 breast tumors with deleterious *BRCA1* mutations were analyzed for Fhit expression by immunohistochemistry in a case-control matched pair analysis [Turner et al., 2002]. Loss of Fhit expression was significantly more frequent in the *BRCA1* cancers compared with sporadic breast tumors (9% Fhit positive vs. 68% Fhit positive), suggesting that the *BRCA1* pathway was also important in protecting the *FRA3B*/*FHIT* locus from damage. To investigate further the relationship between repair gene deficiencies and induction of chromosome fragile sites in vitro, we analyzed the frequency of aphidicolin induction of chromosome gaps and breaks in *PMS2, BRCA1, MSH2, MLH1,* and *FHIT*-deficient cells. Each of the deficient cell lines, including the *FHIT*-deficient, showed elevated expression of aphidicolin-induced chromosome gaps and breaks, consistent with the proposal that proteins involved in mismatch and double-strand break repair are important in maintaining the integrity of common fragile regions. Correspondingly, genes at common fragile sites may sustain elevated levels of DNA damage in cells with deficient DNA repair proteins such as those mutated in several familial cancer syndromes.

To explore the role of the Fhit gene product in stress and DNA damage responses, matched pairs of Fhit-negative and -positive human cancer cell clones, and normal cell lines established from *Fhit*−/− and +/+ mice, were stressed and examined for differences in cell cycle kinetics and survival [Ottey et al., 2004]. A larger fraction of Fhit-negative human cancer cells and *Fhit*−/− murine kidney cells survived treatment with mitomycin C or UVC light compared to matched Fhit-positive cells; 5–10-fold more colonies of Fhit-deficient cells survived high UVC doses in clonigenic assays. After low UVC doses, the rate of DNA synthesis in −/− cells decreased more rapidly and steeply than in +/+ cells, and the Atr-Chk1 pathway appeared over-activated in −/− cells. UVC surviving *Fhit*−/− cells appeared transformed, and exhibited >5-fold increased mutation frequency, an increase that could explain the susceptibility of Fhit-deficient cells in vivo to malignant transformation.

Compared with *Fhit*+/+ cells, *Fhit*−/− cells exhibited an over-activated *ATR*/*CHK1* pathway and showed increased mutation frequency and resistance to DNA damage-induced killing, indicating that Fhit and the Chk1 pathway have opposing roles in cells responding to DNA damage [Hu et al., 2005a]. Hu et al. [2005b] then showed that cells, with or without Fhit expression, have similar DNA double strand break (DSB) induction levels and similar rejoining rates following ionizing radiation. By combining an I-SceI-induced-DNA DSB system and a small interfering RNA approach, it was shown that knocking down Fhit increased the efficiency of homologous recombination repair of cells, but knocking down Chk1 decreased the efficiency of homologous recombination repair

associated with the sensitivity to ionizing radiation-induced killing.

The above results are puzzling for several reasons: (1) If Fhit is involved in a DNA damage response, its loss results in a phenotype opposite to the phenotypes of most proteins in damage response pathways; loss of nearly all such proteins leads to increased sensitivity to DNA damage; (2) it is difficult to imagine how loss of Fhit could lead to a stronger Atr-Chk1 checkpoint and how a stronger checkpoint would lead to increased mutation frequency; (3) homologous recombination should be the error free pathway to DNA damage repair so how would loss of an important gene product, Fhit, lead to increased use of the homologous recombination repair pathway? We will discuss the first two points in more detail in the next section but as for the third point, Sonoda et al. [2006] have recently reviewed studies concerning differential use of the non-homologous end-joining (NHEJ) and homologous recombination repair (HRR) pathways in DSB repair. DSBs arise frequently as a consequence of replication fork stalling and due to the attack of exogenous agents. Despite conservation of the two pathways to DSB repair, from yeast to humans, their relative contribution to DSB repair differs among species. HR plays a dominant role in DSB repair in yeast, while NHEJ contributes significantly to DSB repair in vertebrates, necessitating a regulatory mechanism to choose HRR or NHEJ in vertebrate cells. The nature of the DSB, when it occurs in the cell cycle, and whether there is a sister chromatid for HRR, determines which repair pathway is used. The use of HRR instead of NHEJ at stalled replication forks suggests the requirement for a mechanism for choosing a repair pathway, in addition to the nature of the DSB. Sonoda et al. [2006] summarize the data describing control of the balance between HRR and NHEJ by Poly-{ADP ribose}polymerase (Parp1). In wild type DT40 avian cells, Parp1 was rapidly and transiently activated by a DSB, inhibiting binding of Ku protein (required for NHEJ) and allowing access of HR factors. In the absence of Parp1, the affinity of Ku for DNA increases and access of HR factors is suppressed, leading to a reduction of HRR efficacy. Thus, if Fhit absence enhances HRR, it is possible that the Parp1 level is increased in the absence of Fhit, thereby increasing the access of HR factors and enhancing the HRR pathway, an hypothesis that can be tested. As for how over-activated checkpoint mediated enhanced HRR might adversely affect genomic stability, it has been reported [Richardson et al., 2004] that not all HRR is error-free. Over-expression of Rad51, a homologous recombination and DNA repair protein, led to chromosomal translocations, other chromosome rearrangements, and aneuploidy.

FHIT MODULATES THE DNA DAMAGE CHECKPOINT RESPONSE

In preneoplastic lesions, the DNA damage checkpoint is induced, and loss of heterozygosity at *FRA3B*/*FHIT* precedes or is coincident with activation of the checkpoint response in these early stages. Introduction of exogenous Fhit into cells in vitro leads to modulation of expression of checkpoint proteins Hus1 and Chk1 (see Fig. 2, for summary illustration), at mid-S checkpoint, modulation that led to the induction of apoptosis in esophageal cancer cells, but not in non-cancerous primary cultures. The results suggested that the DNA-damage susceptible *FRA3B*/*FHIT* chromosome fragile region encodes a protein that is necessary for protecting cells from accumulation of DNA damage, through its role in the modulation of checkpoint proteins; and inactivation of Fhit contributes to accumulation of abnormal checkpoint phenotypes in cancer development [Ishii et al., 2006, 2007]. These studies were done mainly using over-expression of Fhit in esophageal cancer cells, through use of Adeno-*FHIT* virus, and raise questions about the normal function of Fhit in the Atr–Chk1 pathway in normal cells. The study showed that when Fhit was down modulated in 293 kidney cells, the level of Hus1 and Rad1 proteins was reduced, and experiments further suggested that Fhit protein stabilizes Hus1 protein possibly through interaction with Rad1, a protein known to be involved in the prevention of Hus1 degradation by the proteasome pathway. Down modulation of Hus1 by siRNA in normal cells apparently caused an increase in phosphoChk1, while over-expression of Fhit led to reduced Chk1 and phosphoChk1 level in the cancer cells. But how and why should the absence of Fhit affect Hus1 or Chk1? If Fhit is involved in the stabilization of these proteins, its role in protein stabilization and/or degradation should be investigated in detail. Certainly the effect of

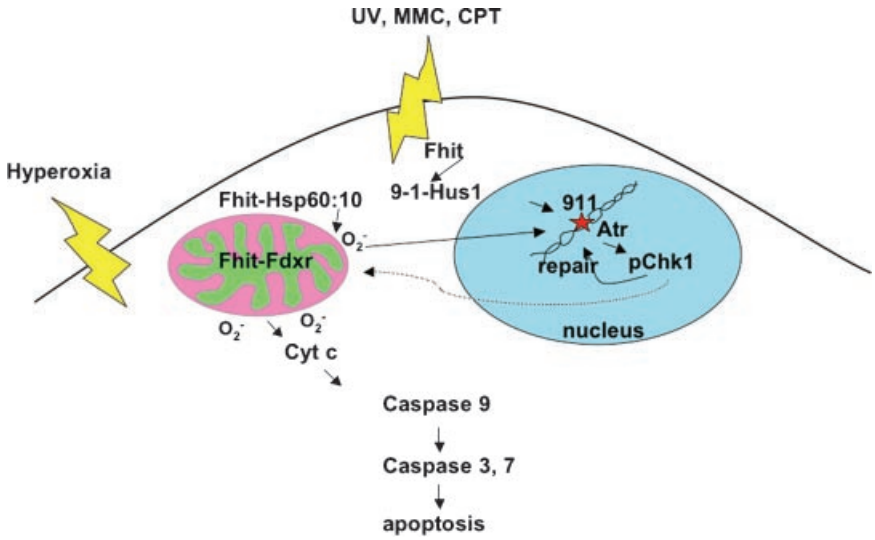

Fig. 2. Roles of Fhit in response to cellular stress. Depiction of the DNA damage and ROS pathways affected by Fhit expression, and possible connections between them. The dashed line from Chk1 in the nucleus to the mitochondrion indicates that if damage is too extensive for repair then apoptosis may be signaled.

Fhit on Hus1 and Chk1 is relevant to the above discussion of an over-activated Atr-Chk1 pathway in Fhit-deficient cells and it will be important to define in detail the mechanisms involved in the Fhit modulation of the Hus1–Chk1 pathway. Currently our focus is on a possible role for Fhit in protein stabilization/destabilization.

FHIT INTERACTION WITH FERREDOXIN REDUCTASE TRIGGERS GENERATION OF REACTIVE OXYGEN SPECIES AND APOPTOSIS OF CANCER CELLS

One way to define specific protein signal pathways is through identification of interacting proteins that could be effectors of function. We have used chemical protein cross-linking and proteomics methods to characterize a Fhit protein complex involved in triggering Fhit-mediated apoptosis [Trapasso et al., 2007]. The complex includes heat shock proteins, Hsp60 and Hsp10, that mediate Fhit import into mitochondria, where it interacts with ferredoxin reductase (Fdxr), a 54 kDa flavoprotein responsible for transferring electrons from Nadph to cytochrome P450 via ferredoxin; electrons can leak from this shuttling system and generate superoxide or reactive oxygen species (ROS) [Hanukoglu et al., 1993]. The Fhit–Fdxr interaction, following mitochondrial import and application of oxidative stress, leads to increased ROS generation, an early event in Fhit-triggered apoptosis; thus, Fhit deficiency leads to oxidative damage to DNA, and survival of cells with higher mutation burdens. Fhit-deficient cancer cells show a mild response to production of ROS, crucial mediators of chemotherapy-induced cell death, confirming that Fhit deficiency could negatively influence treatment outcome. Over-expression of exogenous Fhit significantly increases the sensitivity of lung cancer cells to oxidative stress, leading to enhanced ROS production and apoptosic rate (see Fig. 2 for summary). Thus, characterization of Fhit interacting proteins has identified direct effectors of a Fhit-mediated programmed cell death pathway that is lost in most cancers through loss of Fhit.

Using HCT116 cells with one or three copies of the *FDXR* gene, it was shown that cells with only one copy were less susceptible to Fhit-induced apoptosis and that the level of Fdxr protein was stabilized in the presence of Fhit protein. We are currently determining which proteins in the Fhit complex interact directly, using purified proteins in in vitro binding assays. Additionally, Fhit mutants will be tested for participation in the complex and import to mitochondria, including catalytic site mutants deficient in hydrolysis of Fhit substrate Ap_3A [Trapasso et al., 2003], and FhitY114 mutants [Semba et al., 2006] which cannot be phosphorylated at Y114, as wild type Fhit is by Src family tyrosine kinases. By understanding which proteins of the complex

interact directly and which Fhit mutants may not interact or interact but not cause increased ROS and apoptosis, we will define the cell signal pathways that control Fhit-induced apoptosis and tumor suppression.

Another open question is whether the role of Fhit in the modulation of the Hus1/Chk1 pathway is connected to the role of Fhit in ROS production and apoptosis. We have illustrated possible connections in Figure 2.

While the damaging effects of ROS on DNA have been intensively studied, the effects of oxidative damage on cell cycle checkpoint function have not [Shackelford et al., 2000; Barzilai and Yamamoto, 2004; Macip et al., 2006]. ROS are a significant source of DNA damage under both normal and oxidative stress conditions and play important roles in aging and in diseases, including cancer. Understanding the cell cycle checkpoint responses initiated after oxidative stress may prove useful in understanding the complex role that ROS play in the development of human disease.

Though it is becoming clear that Fhit tumor suppressor functions occur through more than one signal pathway, it will be important to examine possible connections and sequence of activation of specific Fhit signal pathways following exposure to DNA damaging agents or to oxidative stress.

WWOX FUNCTION

Although we do not yet know if the Wwox tumor suppressor protein, encoded by the *FRA16D/WWOX* fragile locus, has a role in the DNA damage or checkpoint responses, we do know that Wwox has a role in induction of apoptosis when over-expressed in breast, lung, and other cancer cells. We also know that Wwox protein, through its N-terminal WW domain, a protein interaction domain, interacts with p73, Jun, ErbB4, and several transcription factors [Iliopoulos et al., 2006; Aqeilan and Croce, 2007]. Wwox has also been reported to interact with p53 [Chang, 2002], so it is possible to imagine that Wwox is part of a network of WW-domain proteins, that by hierarchical competition for specific interactors based on binding affinities, could control responses to stimuli such as DNA damage in specific cell types. A Wwox knockout strain has been developed and characterized; the hemizygous knockout mice are viable, fertile, and more susceptible to the development of spontaneous and induced tumors [Aqeilan and Croce, 2007], while the double knockout mice do not survive long after birth. Cell lines have been established from the knockout mice and the response of these cells to DNA replication stress and other forms of DNA damage will be assessed to determine if Wwox has a role in response to various DNA damaging agents.

PROSPECTS

Delineation of direct downstream effectors of the Fhit suppressor pathway will lead to intensification of mechanistic studies of Fhit function that may influence future preventive and therapeutic strategies to activate the Fhit pathway or to specifically target Fhit-deficient cancers. In addition, this method of identification of elusive Fhit interactors may lead to discovery of Fhit interactors in other cellular compartments, allowing identification of additional Fhit signal pathways with relevance to normal and cancer cell growth control. The finding that ROS generation is crucial for Fhit-mediated apoptosis emphasizes the importance of Fhit loss as a negative prognostic factor in various clinical settings; for example, assessment of Fhit status in preneoplastic or neoplastic conditions may be predictive of responses to antioxidant treatments. Finally the fact that Fhit may need Hsp chaperones to enter mitochondria and initiate its apoptotic pathway suggests that drugs enhancing chaperone expression might have efficacy in preneoplastic or neoplastic conditions associated with Fhit expression.

Wwox and Fhit expression is lost coordinately in breast cancers [Guler et al., 2004, 2005], likely due to the similar susceptibility of FRA3B and FRA16D to DNA damage, so antioxidant treatments that inhibit damage to the FHIT locus would be effective at the WWOX locus. Also, retention of strong expression of Wwox protein is correlated with response of estrogen receptor positive breast cancers to tamoxifen therapy [Guler et al., 2007], so a clearer understanding of the role of Wwox and its interacting proteins in suppressing breast cancer may lead to identification of new prognostic markers and novel therapeutic targets.

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