Minireview

WWOX, the chromosomal fragile site *FRA16D* spanning gene: Its role in metabolism and contribution to cancer

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Abstract

The *WWOX* gene spans the common chromosomal fragile site *FRA16D* that is located within a massive (780 kb) intron. The *WWOX* gene is very long, at 1.1 Mb, which may contribute to the very low abundance of the full-length 1.4 kb mRNA. Alternative splicing also accounts for a variety of aberrant transcripts, most of which are devoid of C-terminal sequences required for WWOX to act as an oxidoreductase. The mouse *WWOX* gene also spans a chromosomal fragile site implying some sort of functional relationship that confers a selective advantage. The encoded protein domains of WWOX are conserved through evolution (between humans and *Drosophila melanogaster*) and include WW domains, an NAD -binding site, short-chain dehydrogenase/reductase enzyme and nuclear compartmentalization signals. This homology has enabled functional analyses in *D. melanogaster* that demonstrate roles for WWOX in reactive oxygen species regulation and metabolism. Indeed the human *WWOX* gene is also responsive to altered metabolism. Cancer cells typically exhibit altered metabolism (Warburg effect). Many cancers exhibit *FRA16D* DNA instability that results in aberrant *WWOX* expression and is associated with poor prognosis for these cancers. It is therefore thought that aberrant *WWOX* expression contributes to the altered metabolism in cancer. In addition, others have found that a specific (low-expression) allele of *WWOX* genotype contributes to cancer predisposition.

Keywords: Chromosomal fragile site, *FRA16D*, oxidoreductase, altered metabolism

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Introduction

WWOX gene spans the common chromosomal fragile site, FRA16D

Chromosomal fragile sites are enigmatic regions of human chromosomes that are of particular interest because of their contribution to human disease (Figure 1). Fragile sites are distinguished by their frequency – *rare* fragile sites are found in only a minority of the population and are inherited, while *common* fragile sites are found in all individuals. Common fragile sites are also distinguished by the chemicals that cause their cytogenetic appearance. The inducing chemicals include agents typically found in the diet (e.g. folate, caffeine, and ethanol[1,2]), as well as in the environment (e.g. chemicals in cigarette smoke[3]). The major group of rare fragile sites are induced by folic acid, while the majority of common fragile sites are induced *in vitro* by aphidicolin, an inhibitor of DNA polymerase. It is this group of common, aphidicolin-inducible fragile sites that are associated with stress during replication and consequent DNA instability. Common fragile sites are also noteworthy because of their frequent colocation with regions of chromosomal instability in cancer.[1,44] There is a hierarchy in the cytogenetic appearance of fragile sites *in vitro* in response to chemical induction and that is matched by their DNA instability observed *in vivo* in various cancers – indicating a direct relationship between cytogenetic appearance and DNA breakage in cancer. This DNA instability appears to be an early event in carcinogenesis occurring as a result of exposure to environmental agents such as the chemicals in cigarette smoke.[3] The two fragile sites, *FRA3B* and *FRA16D*, that are the most readily induced *in vitro* are also those that most frequently exhibit DNA instability *in vivo* in cancer.[2,4,5] Common fragile sites and the genes in their vicinity that are affected by their DNA instability in cancer are therefore of interest for their causal contribution to neoplasia.

WWOX is a protein encoded by a common fragile site gene (Figure 2). The *WWOX* gene spans *FRA16D* that is located within a very large, 780 kb intron of this alternatively spliced, massive gene of 1.1 Mb in length.[4,45] The time taken for transcription of such a length of DNA makes it likely that just one or a few full-length spliced transcripts are produced per cell cycle. As has been found

for several common fragile site genes, the relationship between the *WWOX* gene and a common fragile site is conserved between mouse and human, implying a functional relationship with a selective advantage of some sort. The alternative splicing of the *WWOX* gene transcript leads to an abundance of aberrant transcripts incapable of encoding the full-length WWOX protein.[4] While these appear to be the subject of nonsense-mediated decay, some truncated proteins are produced that may act as dominant negative competitors for the full-length protein.[7] Some of these alternative splice RNA products are conserved between mouse and human, also implying functional significance.

A genetic variant of the *WWOX* locus, that has lower levels of WWOX, has been found to be associated with cancer predisposition. Specifically, carriers of loss variant (CNV-67048) genotypes have been found to have significantly increased risk of lung cancer, in a dose-dependant manner.[8] Recently, a similar association has been found between the CNV-67048 genetic variant and risk of gliomas.[9]

Box 1. Common chromosomal fragile sites (CFSs) and their genes[43].
a) CFSs represent specific and reproducible regions of the genome that are sensitive to environmental stress and sites of DNA damage in cancer.
b) CFS exhibit a hierarchy of cytogenetic appearance *in vitro* that reflects their observed DNA instability *in vivo*.
c) CFS genes are typically very large (>1Mb) and are unrelated in primary sequence.

Figure 1 Summary box 1 – common chromosomal fragile sites and their genes.[43]

Box 2. WWOX in cancer initiation and progression

a) loss of *WWOX* expression correlates with poor prognosis in multiple cancers[18-21]. e.g. Reduced *WWOX* levels correlate with poor prognosis in colorectal cancer.
b) *FRA16D / WWOX* DNA instability is one of the most common recurrent sites of deletion in cancer[4,5,23,44].
c) *WWOX* re-introduced back into *WWOX*-deficient cancer cells suppresses the growth of tumors[6].
d) *WWOX* levels are reduced in 20-50% of a wide variety of cancers particularly specific types of lymphoma and leukemia.
e) *WWOX* mutation is an early event in cancer cell development[2,4,44].
f) *WWOX* CNVs with low WWOX levels predispose to lung cancer[8,9].

Figure 2 Summary box 2 – WWOX in cancer initiation and progression

Characteristics of the WWOX protein sequence

The WWOX protein itself comprises multiple distinct functional domains as well as sequences encoding a small chain dehydrogenase/reductase (SDR) enzyme. Phylogenetic analysis (Figure 3) of the WWOX amino acid sequence indicates evolutionary conservation of WWOX as a discrete ortholog, from species as diverse as humans and insects.[10] The functionally important sequences in WWOX, including the WW domains and the SDR enzyme sequences, are evident as more highly conserved regions of the WWOX sequence (Figure 4). SDR enzymes typically have their specific substrate-binding sequences located C-terminal to the catalytic region. The WWOX orthologs exhibit sequence homology in this WWOX-specific domain that is as high as that for the enzyme co-factor and catalytic regions, consistent with these sequences being the region where WWOX

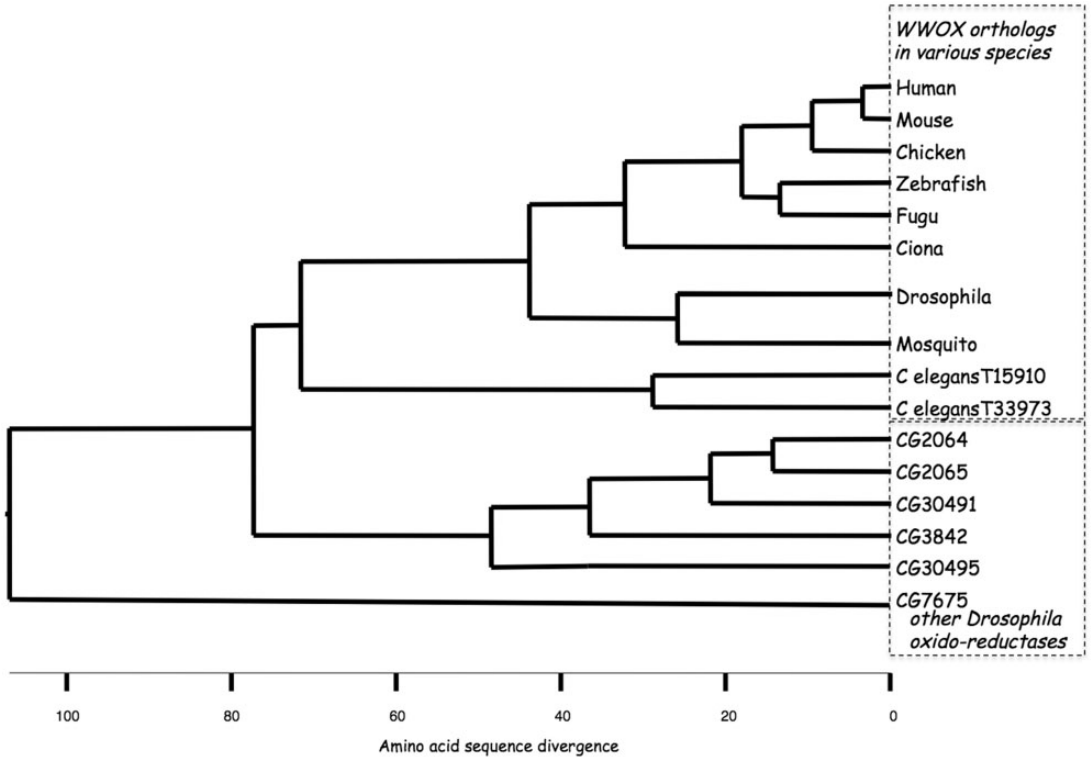

Figure 3 Phylogenetic tree of WWOX orthologs from various species and other oxidoreductases in *Drosophila melanogaster*. Distinct sequence relationships through evolution of WWOX orthologs in various species are evident when compared to other oxidoreductases (in this case from *Drosophila*). *Note*: all species have a single WWOX ortholog except *Caenorhabditis elegans* with two, neither of which has WW domains. Figure modified from that of O'Keefe et al.[10]

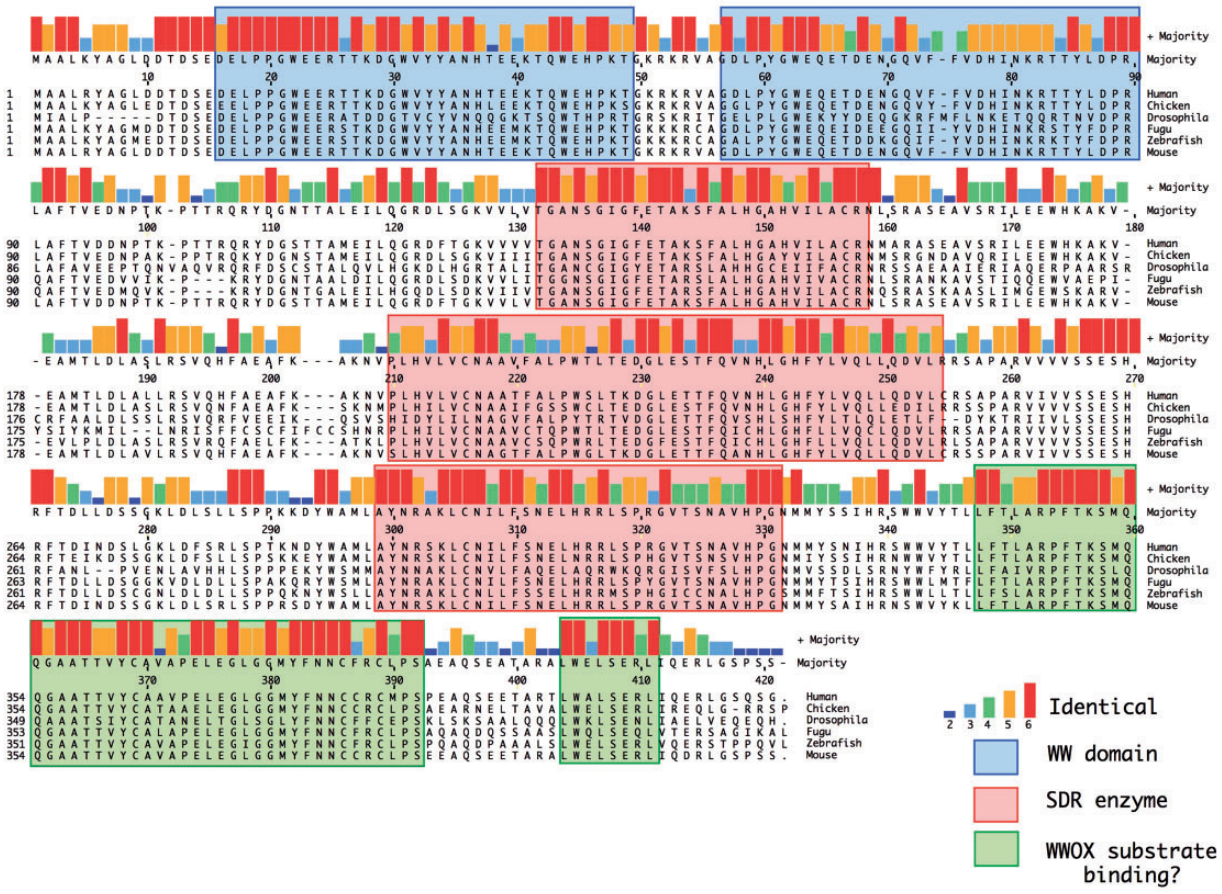

Figure 4 Homology between orthologs WWOX protein sequences. Alignment of WWOX protein sequences from human, chicken, *Drosophila melanogaster*, zebrafish, and mouse. Majority sequence is indicated with color coding for identity between 6, 5, 4, 3, and 2 sequences. The two WW domains are indicated by blue shading. Sequences comprising the SDR enzyme are indicated by red shading, and the putative ''WWOX substrate binding site'' is shaded in green. (A color version of this figure is available in the online journal.)

binds to its specific (as yet unknown) substrate. Each of the SDR enzymes contains an nicotinamide adenine dinucleotide (oxidized form) (NAD^+)/nicotinamide adenine dinucleotide phosphate ($NADP^+$) co-factor-binding site as well as characteristic sequences required for the catalytic active site. The *in vivo* substrate and reaction product for the SDR enzyme activity of WWOX are unknown, although several *in vitro* targets have been identified[11] and ablation of WWOX in mice leads to impaired steroidogenesis.[12] Steroids are therefore likely suspects although impaired steroidogenesis is not evident in the *Drosophila melanogaster* WWOX mutants.

It is noteworthy that the closest organism that does not have a complete WWOX ortholog, *Caenorhabditis elegans,* has two genes with homology to WWOX but both are devoid of the two N-terminal WW domains (Figure 3). However, both have sequence homology to the putative ''WWOX substrate binding region,'' suggesting that these enzymes act on the same substrate/substrates and that this is independent of the WW domain interactions seen in, and presumably needed for, WWOX activity in other species.

The link between the WWOX substrate binding domain and SDR enzyme function appears evident in Opisthokonts (*Casaspora owczarzaki*) and *C. elegans* orthologs not having WW domains but sharing substrate-binding domain homology. The addition of WW domains to WWOX is an ancient event, albeit of unknown biological significance, with the sea sponge (*Amphimedon queenslandica*) WWOX protein having both WW domains and all other WWOX domains, including substrate-binding domain homology (not shown).

WW domains are known to act as protein–protein recognition and binding domains, pairing up with proteins through proline-containing motifs (PPXY or similar). WW-mediated protein interactions are thought to be a physical mechanism that causes a functional relationship between the partner proteins. The identity of the WW-binding motif partners of WWOX has therefore been sought in order to gain insight into the function of WWOX. WW domains have been classified into subgroups with specificity in their interacting motifs, e.g. the WW1 motif of WWOX is expected to interact with PPXY or LPXY motifs. Mass spectrometry and phage display techniques have been used in an effort to identify the specific PPXY-containing WWOX-interacting proteins.[13] This analysis revealed that the WW1 domain of WWOX is capable of binding to many different proteins, although the significance of most of these interactions to the function of WWOX is yet to be determined.

In addition to the WW domains, the WWOX protein contains compartmentalization signals for localization to the nucleus and the mitochondria, although the latter is not well defined.[14,15] The SDR-related sequences define WWOX as a member of this family of enzymes. These enzymes are NAD(P)(H)-dependent oxidoreductases that catalyze diverse small molecule reactions including those involving lipids, amino acids, carbohydrate, and steroids.[16] NAD(P)(H) binding is not only for the role of this co-factor in catalysis but also as a means for SDR enzymes to act as metabolic sensors of NAD^+/nicotinamide adenine dinucleotide (reduced form) (NADH)/$NADP^+$/NADP levels and in so doing contribute to the regulation of metabolism, transcription, and signal transduction. The typical small molecule substrates and products of SDRs contribute to their designation as a ''druggable'' enzyme class, suitable for targeting with potential pharmaceuticals.

WWOX is able to act as a ''non-classical'' tumor suppressor

When cancer cells exhibit DNA instability at the *FRA16D* site, this is typically associated with a reduction in full-length WWOX mRNA and an increase in either normal incomplete splice forms and/or the appearance of novel aberrant transcripts. While the resultant cancer cells therefore have lower levels of WWOX, they do not exhibit second allele loss typical of Knudsen's two-hit hypothesis and therefore retain some level of WWOX protein. Even so the transfer of intact WWOX gene back into tumor cells that are deficient in this protein, does reduce the ability of these WWOX-replenished cells to form tumours[6] and therefore WWOX is regarded as a tumor suppressor, albeit a ''non-classical'' one – i.e. a reduction in WWOX level, rather than its absence, appears sufficient for its functional contribution to cancer. The mechanism by which WWOX might act in this capacity is unclear although some possibilities have been put forward on the basis of the proteins with which WWOX physically interacts. For example, the WW domains of WWOX may serve as negatively regulating, competitor-binding sites for proteins such as ErbB-4, thereby competing with these proteins for their ability to physically and functionally interact with other WW domain-containing proteins, in particular yes-associated protein (YAP).[17] Reduction in WWOX levels below a threshold may therefore facilitate the activation by YAP of tumor-promoting pathways mediated by ErbB-4 and perhaps other proteins.

Consistent with its role as a tumor suppressor, patients with tumors who exhibit diminished levels of WWOX, have a poorer prognosis than those with normal levels of the protein. Nunez et al.[18] reported that WWOX protein expression varies among ovarian carcinoma histotypes and correlates with less-favorable outcome. In addition, Pluciennik et al.[19] found that *WWOX* expression correlated with breast cancer progression and prognosis. Furthermore, Aqeilan et al.[20] and Wang et al.[21] reported the prognostic significance of WWOX expression levels in patients with breast cancer and its association with the basal-like phenotype. The reduction in WWOX levels, by DNA instability at one allele, appears to be sufficient to enable a functional contribution of some sort to cancer cell biology. The normal biological function of WWOX is therefore of particular interest as it represents a target for improving the prospects of individuals with WWOX deficient cancers.

FRA16D/WWOX damage is an early event in a population of precancerous cells

A clear illustration of the mechanism through which environmental factors are thought to trigger cancer through effects on common fragile sites, is the contribution of cigarette smoke to fragile site DNA instability in lung cancer.[3] Much greater levels of cytogenetic fragile site expression are well documented in the ''normal'' cells of young cigarette smokers, well before the onset of lung cancer.[22] There is a direct correlation between *in vitro*-induced cytogenetic fragile site expression and DNA instability observed as a consequence of events *in vivo*.[2,23] The influence of tobacco smoke chemical exposure on fragile site expression is followed by chromosome breakage and/or rearrangement that, in turn, contributes to carcinogenesis. The deleterious ''hit'' related to small cell lung cancer may occur at any time during tobacco exposure, but, given that carcinogenesis is a stepwise process, the actual tumor may not arise for several years after the individual stops smoking.[3] Such ''fragile site damaged'' cells will not exist in isolation but as a population of similarly ''damaged'' cells. Cigarette smoke chemicals preferentially affect the most readily induced common fragile sites (*FRA3B* and *FRA16D*) and, therefore, common fragile site genes (*FHIT* and *WWOX*). Any one of these *FRA16D/WWOX* damaged cells has the potential to act as a cancer stem cell (CSC), particularly after additional damage in genes that also contribute to cancer initiation. We have found that common fragile site damage at *FRA16D* is an early event in cancer cell progression.[23] Two cancer cell lines developed from a primary carcinoma (KM12C) and secondary metastasis (KM12SM) have identical *FRA16D* deletions despite having drastic differences in their karyotype.

The normal biological function/functions of WWOX

The biological function of *WWOX* has been explored by genetic approaches, both by targeted mutagenesis and by the identification and characterization of spontaneous *WWOX* mutations in a rat, and more recently, in humans. A ''non-classical'' tumor suppressor function for *WWOX* is evident in the analysis of rodent mutants for the *WWOX* gene. Some (but not all) lines of loss-of-function *WWOX* mutant mice have higher incidence of tumors, however tumors from heterozygous mutant mice still express WWOX.[24–27] A spontaneous *WWOX* mutant rat does not exhibit higher incidence of tumors.[28,29] Rodent *WWOX* mutants typically exhibit metabolic disorders leading to early death (which may preclude development of some tumors) limiting the utility of rodent models in understanding WWOX protein function.[24–29] While these rodent studies have provided some insight into the biological role for WWOX, there is still clearly a need to understand the molecular processes and pathways in which WWOX

participates; particularly how WWOX contributes to metabolism and how this altered metabolism can contribute, at least in certain circumstances, to cancer.

Three reports detail clinical consequences in humans due to inherited mutations in *WWOX*. The first of these[30] involves a disorder of sex development due to a deletion, in one allele of the *WWOX* gene, removing exons 6–8, with exon 5 being spliced on to exon 9. The resultant encoded protein would have intact WW domains, but absence of the SDR catalytic domain. Only one allele is affected and is therefore acting in a dominant manner. While haploinsufficiency is possible, another explanation is that the mutant WWOX-truncated protein is acting in a dominant negative manner. Two recent reports[31,32] detail recessive epilepsy and other symptoms due to loss of function mutations in both alleles of the *WWOX* gene. Three different homozygous *WWOX* mutations were identified in three consanguineous families. The neurological symptoms in each of the affected individuals are similar to those observed for the spontaneous rat *WWOX* mutation indicating likely common pathogenic pathway for these symptoms. The human and rat *WWOX* mutations also share a lack of noted increase in spontaneous tumours[28,29,31,32] as has been reported for mouse *WWOX* mutations.[24–27]

Insights into the role of WWOX in metabolism

Given the phenotypic consequences of homozygous loss-of-function *WWOX* mutations in mammals, it is somewhat surprising that loss-of-function *WWOX* mutations in the *D. melanogaster* ortholog of *WWOX* do not exhibit a phenotype.[33] The *D. melanogaster* ortholog has 49% amino acid sequence identity with its human counterpart, with all of the functional domains and SDR enzyme-related sequences retained (Figures 3 and 4). The function of WWOX protein in *D. melanogaster* has become evident from the conduct of genetic and biochemical analyses in WWOX-deficient flies. Microarray and proteomic experiments identified quantitative and qualitative changes in other proteins consistent with a role for WWOX in metabolism. An advantage of the *D. melanogaster* system is the ability to undertake genetic analyses of function and this revealed a role for WWOX in pathways that included the proteins superoxide dismutase (SOD1) and isocitrate dehydrogenase (IDH).[33] SOD1 has a role in the control of reactive oxygen species (ROS) in cells, while IDH is an integral component of the citric acid cycle. Both the viability and life span of SOD1 mutant *D. melanogaster* were impacted by altered WWOX levels, while both *D. melanogaster* and human cancer cells showed a correlation between SOD1 and WWOX mRNA levels.[33] Reducing IDH levels in *D. melanogaster* impacts on viability and this impact is exacerbated by also reducing WWOX levels and relieved by increasing WWOX levels.[33] Furthermore, WWOX and IDH mRNA levels correlate in cancer cells.[33] Roles for WWOX in regulation of ROS and metabolism are both intriguing, in terms of the possible mechanism of WWOX contributing to cancer. ROS levels are lower in at least certain types of CSCs than corresponding nontumourigenic cells.[34] These lower ROS levels in CSCs are associated with increased free radical scavenging and decreased sensitivity to ionizing radiation. In this setting, the lower level of ROS brought about by reduced WWOX could therefore contribute to tumor radioresistance, and therefore poorer prognosis for patients with WWOX-depleted cancers.

Altered cellular metabolism in cancer cells was first discovered over 80 years ago by Warburg,[35] but has only recently been added to the list of recognized hallmarks of cancer.[36] Warburg revealed that cancer cells favor adenosine triphosphate (ATP) production via rapid consumption of glucose and formation of lactic acid rather than via the more efficient mitochondrial respiration, even when oxygen is available.[35] There are clear instances of altered metabolism playing causal roles in the development of cancer e.g. mitochondrial DNA mutations in cancers of the head and neck[37] and the recent finding of very specific leukemia-associated *IDH1* and *IDH2* mutations lead to neomorphic enzyme activity, converting alpha-ketoglutarate to the ''oncometabolite'' 2-hydroxyglutarate.[38] Although this change in metabolism has been exploited in the development of drugs for cancer therapy,[39] until very recently, it has been unclear what causal cellular changes bring about this phenotype. Therefore with few exceptions, therapeutics have not yet specifically targeted the causal changes in cancer cells that are responsible for altered cellular metabolism. There is, however, growing recognition that because of this distinction between normal and cancer cells, altered metabolism has the potential for such exploitation.[40,41] Thus the molecular cause/causes of altered metabolism in cancer cells need/needs to be defined in order to enable the identification of therapeutic leads.

WWOX not only contributes to metabolic regulation, its expression is also responsive to changes in metabolism.[42] Altering metabolism from glycolysis to oxidative phosphorylation causes an increase in the steady-state levels of WWOX mRNA, whereas hypoxic conditions, in which cells rely on glycolysis, cause a decrease in WWOX mRNA. Both the *WWOX* gene and its encoded protein are therefore monitors of and contributors to the metabolic state of cells.

Perturbation of WWOX in cancer cells is therefore a clear candidate for contributing to the altered metabolism (Warburg effect[35]) seen in these cells and associated with poor prognosis, and therefore an ideal target for therapeutic intervention.

Conclusions/future directions

The presence of a distinct WWOX protein, in species as diverse and distant in evolution as sea sponge and humans, would suggest that it serves a fundamental function in biology. Indeed the protein has normal roles in metabolism. The level of WWOX protein is affected by DNA instability within its gene in human cancers. Reduced WWOX level appears to facilitate cancer progression, rather than act as a classical tumor suppressor. Lower levels of WWOX, due to genetic variation, contribute to the risk of carriers to increased cancer incidence. Lower levels of WWOX in cancers correlate with poorer prognosis.

Clearly in both cases, mutations in other genes are required to both initiate and establish cancer.

The altered metabolism of cancer cells is an intriguing possibility for the contribution of WWOX to cancer cell biology. Identifying the role of WWOX in metabolism and targeting this role in cancers found to be deficient in WWOX may lead to better therapeutic interventions.

The WW domains of WWOX have been the focus of attention as the identity of their partner proteins can give insight into the pathways to which WWOX contributes. However, the role of WWOX in metabolism is more likely to be mediated by the enzyme function of WWOX, as SDR enzymes are known monitors of $NAD^+/NADP^+$ through their co-factor binding sites.[16] Indeed the *C. elegans* orthologs of WWOX do not contain WW domains. The substrate and product of WWOX are therefore of particular interest. Protein homology searches indicate that the likely ''substrate-binding domain'' indicated by amino acid sequence homology C-terminal to the enzyme catalytic site (Figure 4) appears to be unique to WWOX orthologs (including those of *C. elegans*) and therefore likely to have a unique substrate. Either the accumulation of substrate or the reduced level of product of the WWOX enzyme, present potential targets to compensate for the contribution that lower WWOX levels, which lead to increased cancer risk and/or poorer cancer prognosis.

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