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Review

WVVOX at the crossroads of cancer, metabolic syndrome related traits and CNS pathologies

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ABSTRACT

WVVOX was cloned as a putative tumor suppressor gene mapping to chromosomal fragile site FRA16D. Deletions affecting WVVOX accompanied by loss of expression are frequent in various epithelial cancers. Translocations and deletions affecting WVVOX are also common in multiple myeloma and are associated with worse prognosis. Met-analysis of gene expression datasets demonstrates that low WVVOX expression is significantly associated with shorter relapse-free survival in ovarian and breast cancer patients. Although somatic mutations affecting WVVOX are not frequent, analysis of TCGA tumor datasets led to identifying 44 novel mutations in various tumor types. The highest frequencies of mutations were found in head and neck cancers and uterine and gastric adenocarcinomas.

Mouse models of gene ablation led us to conclude that *Wvvox* does not behave as a highly penetrant, classical tumor suppressor gene since its deletion is not tumorigenic in most models and its role is more likely to be of relevance in tumor progression rather than in initiation. Analysis of signaling pathways associated with WVVOX expression confirmed previous *in vivo* and *in vitro* observations linking WVVOX function with the TGFβ/SMAD and WNT signaling pathways and with specific metabolic processes. Supporting these conclusions recently we demonstrated that indeed WVVOX behaves as a modulator of TGFβ/SMAD signaling by binding and sequestering SMAD3 in the cytoplasmic compartment. As a consequence progressive loss of WVVOX expression in advanced breast cancer would contribute to the pro-metastatic effects resulting from TGFβ/SMAD3 hyperactive signaling in breast cancer.

Recently, GWAS and resequencing studies have linked the WVVOX locus with familial dyslipidemias and metabolic syndrome related traits. Indeed, gene expression studies in liver conditional KO mice confirmed an association between WVVOX expression and lipid metabolism.

Finally, very recently the first human pedigrees with probands carrying homozygous germline loss of function WVVOX mutations have been identified. These patients are characterized by severe CNS related pathology that includes epilepsy, ataxia and mental retardation.

In summary, WVVOX is a highly conserved and tightly regulated gene throughout evolution and when defective or deregulated the consequences are important and deleterious as demonstrated by its association not only with poor prognosis in cancer but also with other important human pathologies such as metabolic syndrome and CNS related pathologic conditions.

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1. Introduction

By using conventional positional cloning approaches in 2000 we cloned WWOX (WW domain containing oxidoreductase), a gene spanning chromosome region 16q23.3–q24.1, an area that was narrowed down as commonly affected by genomic losses in breast and other cancers [1,2]. WWOX contains two distinct domains; the amino terminus shows high sequence conservation to the WW domain family of proteins displaying two WW domains and a central 283 amino acids domain homologous to the Short-Chain Dehydrogenase/Reductase (SDR) superfamily (Fig. 1).

At the genomic level WWOX spans a region of over 1.1 Mbp in size even though the encoded gene is not large, only 9 exons (NM_016373.2) coding for a 414 AA protein (NP_057457.1). We observed that the very large 5 and 8 introns contain the previously identified translocation break points for multiple myeloma, specifically t(14;16)(q32;q23) [3]. We concluded that such intronic regions were highly recombinogenic and prone to chromosomal rearrangements and we speculated in our report that likely this was the same locus as that of chromosomal fragile site FRA16D [2]. Soon thereafter a second group in search of the gene target for FRA16D cloned FOR that resulted to be the same as WWOX confirming the original speculations [4] and a third group reported the mouse sequence [5]. FRA16D, is the second most frequently affected constitutive site of chromosomal fragility in the whole human genome, second only to FRA3B. Common or constitutive chromosomal fragile sites are found in all individuals and are a constant chromosomal feature. These highly recombinogenic chromosomal loci are susceptible to the occurrence of breaks, gaps and rearrangements.

2. WWOX throughout evolution

There is remarkable sequence conservation both at exonic and intronic levels at the human and mouse orthologous chromosomal fragile site regions for human FRA16D/WWOX and mouse Fra8E1/Wwox [6]. WWOX/Wwox proteins display 94% identity and 96% similarity at the amino acid level [5,6]. Remarkably when comparing with the drosophila homologous protein, identity reaches 49% and 66% similarity. Interestingly, within WWOX introns there is remarkable evolutionary conservation, multiple ultraconserved regions can be found within these intronic regions suggesting that there must exist significant evolutionary selection pressure for preserving not only the coding but also the non-coding regions of the WWOX locus. This phenomenon that is also observed in other large fragile site loci such as FRA3B/FHIT, appears

to be partially related to sequences encoding essential non-coding RNAs within such large intronic regions [7,8]. An additional reason for the preservation of such large non-coding areas at the WWOX locus is the proven existence of various functional enhancer elements within such intragenic regions [9].

Gilad et al. [10] utilized a multispecies cDNA array containing probes for >1000 genes matching human, chimpanzee, orangutan and rhesus macaque sequences, thus phylogenetically representing approximately 70 Ma of evolution, for comparing liver specific gene expression between these species. One of the goals of the study was to identify those genes whose expression has remained constant throughout evolution, i.e. genes that remained evolving under 'stabilizing selection'. Interestingly, WWOX ranked among the top genes under stabilizing selection, a category mostly enriched by genes that fall under the gene ontology functional classification of 'regulation of physiological processes'. Furthermore, these authors concluded that precisely focusing on genes such as WWOX, whose expression is tightly conserved among primates, would be helpful for identifying promising candidates for disease-association studies [10].

3. WWOX normal expression and loss of expression in cancer

We observed that WWOX protein is ubiquitously expressed in human tissues by means of immunohistochemistry (IHC) studies [11]. However it is preferentially highly expressed in secretory epithelia, in reproductive, exocrine and endocrine organs and interestingly also in neuronal bodies throughout the CNS including strong expression in the Purkinje layer of the cerebellum [11]. RNASeq analyses from multiple human tissues are quite in agreement with the previous IHC studies and thyroid, brain, testis, ovary and prostate demonstrated to be the tissues with the highest expression levels in that order (Fig. 2) as per the EMBL-EBI Expression Atlas (<http://www.ebi.ac.uk>) Illumina Body Map (experiment E-MTAB-513).

Studies from various laboratories have shown loss of WWOX protein expression in multiple neoplasias such as breast [12,13], ovarian [14], gastric [15], liver [16], lung [17], thyroid [18], pancreatic [19] and other cancers [20]. This topic has been reviewed extensively elsewhere by other authors [21]. Importantly, in various cancers loss of WWOX expression is associated with indicators of poor prognosis and outcome, e.g. in breast cancer [12,13], ovarian cancer [14], non-small cell lung cancer [17] and bladder cancer [22].

It is worth noting that various alternatively spliced mRNA WWOX forms were originally described by us and others as commonly



Fig. 1. WWOX protein domains. Protein-protein interaction WW domains are shown in blue, conserved tryptophans (W) are highlighted. In the second WW domain one tryptophan is replaced by a tyrosine (conservative change). The highly conserved short chain dehydrogenase domain (SDR) is shown in orange. The characteristic oxidoreductase catalytic active triad is highlighted with S281 and the motif YXXXK noted at position 293–297. The conserved coenzyme binding region is also shown TGXXXGXXG at position 130–137. Described phosphorylation sites at Y33 and Y287 are not depicted.

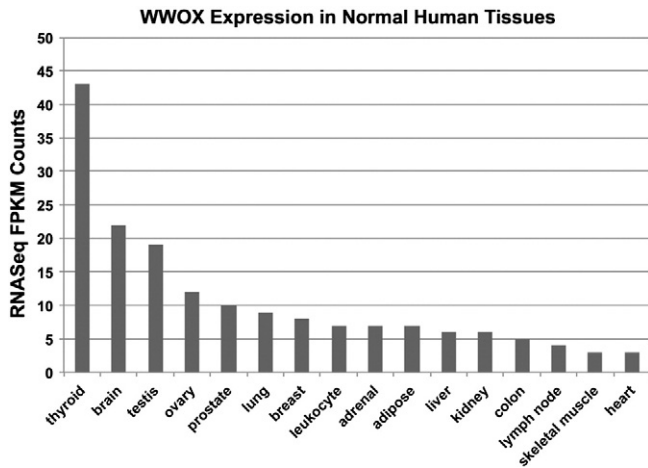


Fig. 2. WFOX expression in normal human tissues as per RNASeq analyses. Data obtained from the EMBL-EBI Expression Atlas (<http://www.ebi.ac.uk>) Illumina Body Map (experiment E-MTAB-513). FPKM: Fragments per kilobase of exon per million fragments mapped.

occurring in tumors and cancer lines. The most common of these alternative transcripts displayed deletions of exons 5–8 or 6–8 [4,23,24]. However, these alternatively spliced forms and others including those reported in GeneBank as isoforms 2 (NM_130791.2) and 3 (NM_130844.2) so far have not been demonstrated to encode viable protein, since they are not detected by Western blot to the best of our knowledge.

The most important mechanism leading to loss of WFOX expression appears to be genomic loss via gross chromosomal deletions and rearrangements as will be discussed in the next section. Additional mechanisms include epigenetic silencing by promoter hypermethylation [25] and degradation. It has been shown that WFOX can be inactivated via polyubiquitination and subsequent proteosomal degradation. ACK1 has been shown to interact with and phosphorylate WFOX at Y287 thus targeting WFOX for rapid degradation [26]. WFOX has also been proposed to be a substrate of ubiquitin ligase ITCH [27]. Additionally recently WFOX downregulation via targeting by miR-134 in head and neck squamous cell carcinomas has also been reported [28].

4. New data on WFOX copy number losses and mRNA expression in cancer

A comprehensive analysis of copy number variation on over 740 cancer lines determined that the WFOX locus (FRA16D) was the third most common site of the whole genome affected by hemi and homozygous losses only after the p16 (CDKN2A) and the FHIT (FRA3B) loci [29]. Recently, Beroukhim R et al. [30] reported a high resolution study of somatic copy number alterations analyzing over 3000 cancer specimens and identified WFOX among the most commonly affected genes by genomic deletions as per GISTIC (genomic identification of significant targets in cancer) algorithm analyses [31]. By using the same algorithm we can now determine genomic losses on each specific tumor dataset reported so far by The Cancer Genome Atlas (TCGA) using the cBioPortal resource (www.cbioportal.org) [32,33]. Thus we identified the tumor types most commonly affected by heterozygous and homozygous WFOX deletions (Freeze February 2014). As can be observed in Fig. 3, ovarian serous cystadenocarcinoma is the tumor type most commonly affected by genomic losses affecting WFOX with 78% of cases showing heterozygous loss and 6% homozygous losses [34] Heterozygous losses were observed in 52% of breast cancers [35], 33% of gastric adenocarcinomas, 29% of prostate adenocarcinomas, 21% of endometrial cancers [36] and 10% colorectal carcinomas [37] (Fig. 3). Gastric adenocarcinomas and prostate carcinomas show a

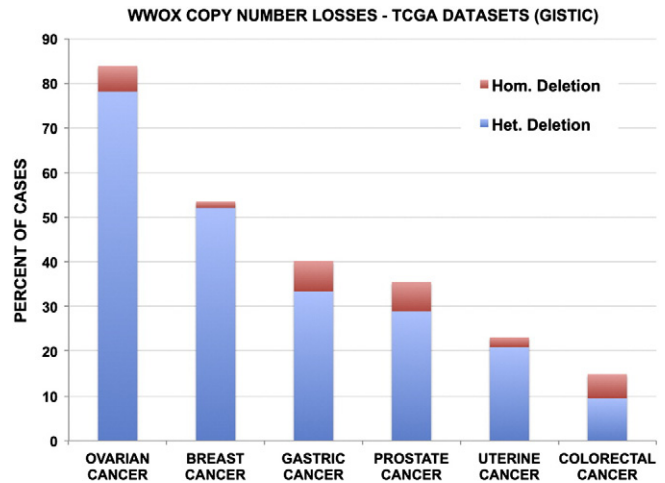


Fig. 3. Copy-number losses affecting the WFOX locus on TCGA tumor datasets, GISTIC algorithm [31], data obtained from www.cbioportal.org. Freeze from February 2014. Ovarian serous cystadenocarcinoma, n = 489 (34); breast cancer, n = 778 (35) and TCGA provisional data); stomach cancer n = 293, (TCGA provisional data); prostate cancer, n = 197 (TCGA tumor set, provisional data), uterine cancer, n=363 (36); colorectal adenocarcinoma, (37 and TCGA provisional data). In red homozygous deletions, in blue heterozygous losses.

relatively high frequency of homozygous losses affecting 7% of cases. Fig. 4 displays the correlation between WFOX copy number status and gene expression levels in ovarian and breast cancer. As can be observed, heterozygous losses affect significantly WFOX expression levels in both tumor types but even more significantly in ovarian cancer.

To further explore the prognostic value of WFOX mRNA expression in breast and ovarian carcinomas, we evaluated information from publicly available gene expression data sets (oligo-microarrays) using the Kaplan–Meier Plotter resource [38]. First, patients were divided into two groups (high and low WFOX expression) according to the quantile expressions of Affymetrix 219077_s_at and 210695_s_at probes. These groups were then compared using the relapse-free survival and progression-free survival for breast and ovarian cancer datasets respectively. Kaplan–Meier analysis revealed that low expression of WFOX was associated with shorter relapse-free survival in breast cancer patients (n = 3259) (Fig. 5) and shorter progression-free survival in ovarian cancer patients as well (n = 1156) (Fig. 6). Interestingly, we identified a significant association between low expression of WFOX and short time relapse-free survival in all breast cancer intrinsic subtypes, but more pronounced in luminal B and basal-like subtypes (Fig. 5).

5. WFOX mutations in cancer

The development of next generation sequencing technologies led to a dramatic accumulation of genomic and expression data in the past three years on multiple tumor types. The data by TCGA gives us now the opportunity to determine the true frequency of somatic mutations affecting WFOX in cancer. Data was obtained using the cBioportal resource (www.cbioportal.org) [32,33], as per freeze in February 2014.

The frequency of mutations affecting the WFOX coding region across tumor types as expected is not very high. Nevertheless a total of 44 novel WFOX somatic mutations have been identified to date in various tumor types, 5 of these resulted in nonsense mutations (3 in head and neck cancers and 2 in lung cancers), 2 frameshift insertions, 1 frameshift deletion and 36 missense mutations, each specific mutation is depicted in Fig. 7 and described in Table 1. Of these, 8 somatic mutations were identified in uterine adenocarcinomas (3.2% of cases, [36], 8 in head and neck cancers (4.1% of cases from Broad [39], and in 2% of TCGA cases), 4 in gastric adenocarcinomas (2.3% of cases), 5 in colorectal cancers (2.2% of cases [37], 6 in melanoma (<2%), 5 in

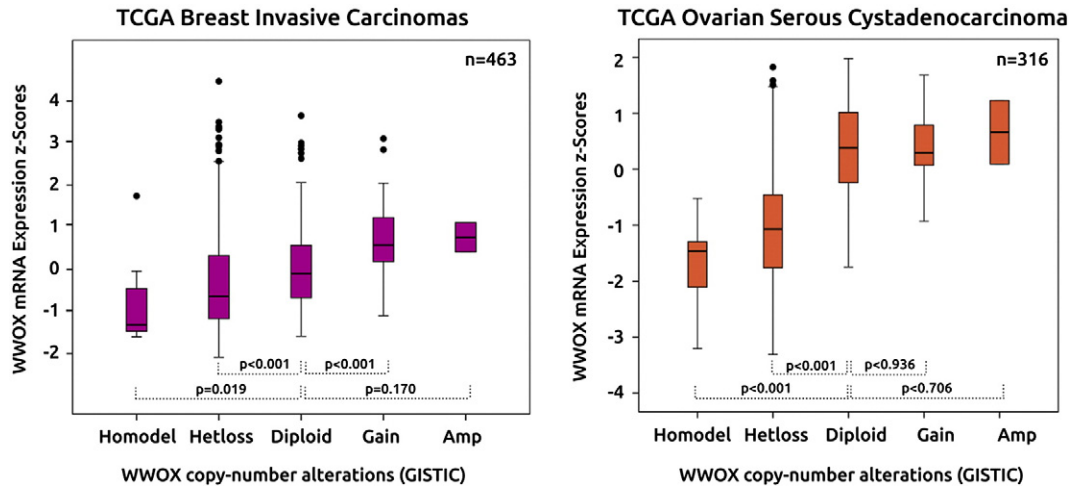


Fig. 4. Correlation plots for WWOX copy-number alterations and corresponding mRNA expression in the TCGA breast cancer (left panel) and ovarian cancer (right panel) datasets as provided by cBioPortal resource. Breast and ovarian samples with WWOX deletion (homozygous and heterozygous deletions) have markedly decreased WWOX mRNA expression compared with diploid samples ($p < 0.05$). WWOX mRNA expression among the copy-number alteration groups was compared with diploid groups using the Mann-Whitney U-Test.

lung adenocarcinomas (<2%), 2 in lung squamous cell carcinomas (<2%), 3 in breast carcinomas (<1%), and one mutation each in a pancreas, a bladder and a renal clear cell carcinoma (Table 1). Of particular interest is that two identical mutations were identified at G260 resulting in frameshift insertions in a uterine adenocarcinoma and in a breast carcinoma, two missense mutations were detected at R254 one in a colorectal and one in a uterine adenocarcinoma (R254C and R254S respectively), two mutations at H147 were also observed

resulting in missense mutations in a head and neck carcinoma and a lung adenocarcinoma (H147Q and H147Y respectively).

The detected nonsense and frameshift mutations all have obvious severe impact in protein functionality and of the 36 missense mutations 18 (50%) are predicted to affect protein function based on the mutation assessor algorithm [40], noted in bold as high and medium functional impact in Table 1. In summary, of all somatic mutations detected so far affecting WWOX in human tumors 59% (26 of 44) are very likely to

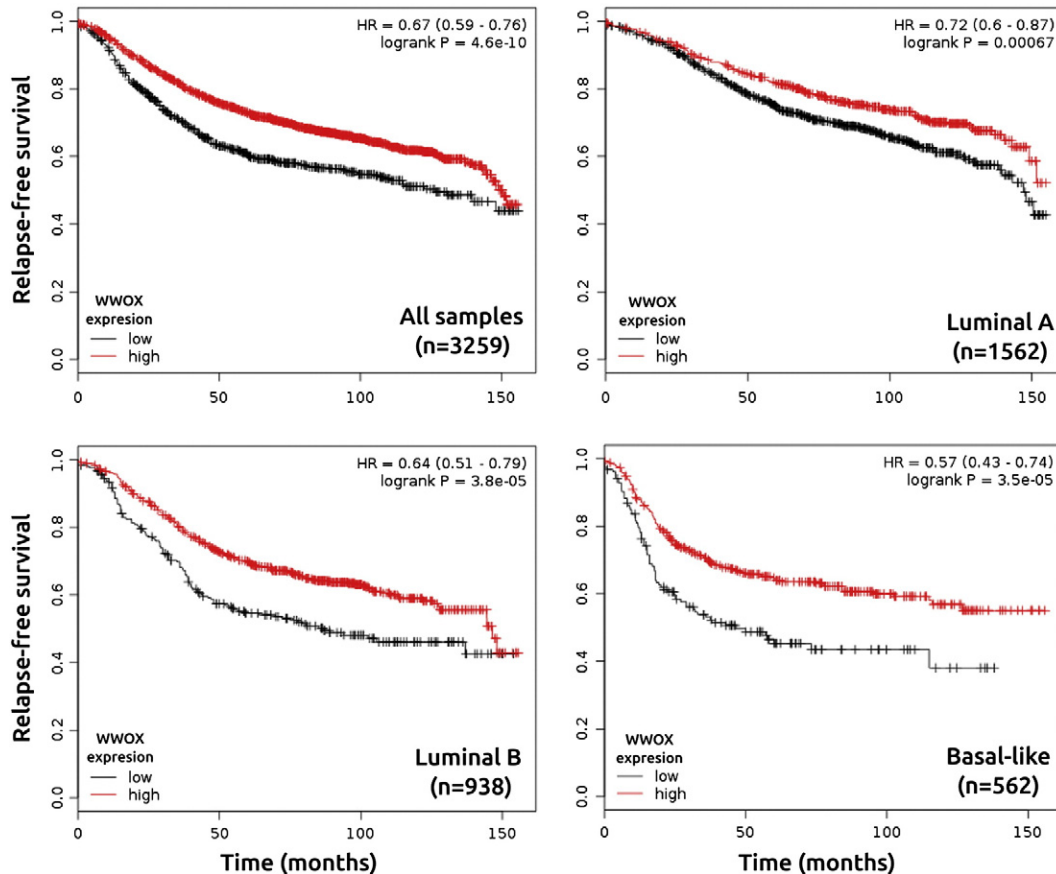


Fig. 5. Survival analysis plots depicting the worse prognostic effect on relapse-free survival of low WWOX expression in breast cancer patients ($n = 3259$) according to each intrinsic subtypes.

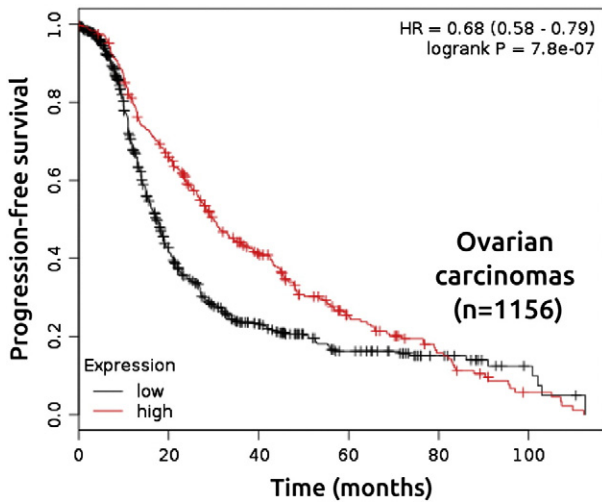


Fig. 6. Survival analysis plots depicting the worse prognostic effect on relapse-free survival of low WWOX expression in ovarian cancer patients ($n = 1156$).

affect protein function, suggesting that is unlikely these mutations represent simple background mutational events.

6. WWOX in multiple myeloma

The frequent involvement of WWOX genomic and expression abnormalities in multiple myeloma (MM) deserves a separate section. In 1998, prior to the cloning of WWOX, Chesi et al. [3], reported the molecular characterization of a novel translocation in MM, t(14;16)(q32;q23). The 16q23 region was observed translocated to an IgH locus in 20–25% of MM lines and fresh MM samples. It was concluded then that the consequence of the t(14;16)(q32;q23) is mostly directed to increase MAF expression (an oncogene located 3' of WWOX's locus) [3]. However, we know now that an additional consequence of such translocation is the destruction of a functional WWOX allele. Analysis of the genomic area spanned by WWOX revealed that the t(14;16)(q32;q23) translocation breakpoints identified as KMS11, MM.1, JN3 and ANBL6 in ch16q23 all map within intron 8 of the WWOX gene [2,6,41]. Translocation t(14;16)(q32;q23) is an indicator of poor prognosis and usually associates with monosomy 13 [42]. Further pointing to the highly recombinogenic nature of the WWOX locus, very recently the generation of chimeric transcripts involving the WWOX and the PVT1 gene (a gene next to the MYC locus) in the RPMI8226 cell line harboring der(16)t(16;22)ins(16;8)(q23;q24), in which PVT1 exon 1 was fused to WWOX exon 9 was reported [43]. Additionally deletion of the WWOX locus is observed in a significant percentage of MM patients (20–35%), as determined by array CGH studies [44] and SNP arrays [45–47]. Furthermore, it was determined that del(16q) was an independent adverse prognostic indicator [45]. It was further determined that LOH at 16q23.1 was significantly associated with loss of WWOX expression in MM cases [46]. Dickens and coworkers characterized regions of homozygous deletion in MM and WWOX was one of 29 genes identified

as affected by homozygous deletions in significant numbers of MM cases [48].

In summary, loss of WWOX expression in MM is frequent and occurs as the consequence of multiple mechanisms including gross chromosomal rearrangements such as deletion and translocations. Additionally, t(14;16)(q32;q23) and 16q losses are both associated with poor prognosis in this disease.

7. Mouse models of WWOX ablation

Since the expression of WWOX is lost in many types of cancer, mouse models of Wwox ablation have been generated to mimic this loss and characterize the subsequent effects on development and tumorigenesis. To this end, a mouse Wwox hypomorphic model [49] and two full knockout lines [50,51] have been generated by different laboratories. The Wwox hypomorphic model was generated by means of gene trap insertion technology targeting the Wwox gene (Wwox^{gt/gt}). Wwox expression was undetectable in embryos (10.5 dpc) and most adult tissues. However, this model was deemed a functional hypomorph because low levels of protein expression could be detected in some tissues such as testes [49]. Phenotypic analysis revealed that Wwox^{gt/gt} mice were viable but had a significantly shorter lifespan than their wild type (WT) counterparts ($p = 0.02$). During the first 18 months of life 23% of Wwox^{gt/gt} died compared to WT and it was not possible to determine the causes of death. However, at the end of the survival experiment Wwox^{gt/gt} female hypomorphs displayed a significant increase in malignant neoplasias compared to WT littermates ($p = 0.015$), with the majority of tumors consisting of B-cell lymphomas. Reduced fertility was also observed due to testicular atrophy in hypomorphic males [49].

Two full Wwox knockout mouse models have been generated either using a targeting construct to delete large regions of the Wwox gene [50] or using mice harboring loxP sites flanking exon 1 of the gene (Wwox^{fl/fl}) crossed to mice expressing Cre-recombinase under the control of the adenovirus E1A promoter (E1A-Cre) [51]. Both full knockout models displayed complete ablation of Wwox protein in all tissues examined with heterozygotes expressing ~50% of WT levels. It was also found that Wwox KO mice die postnatally as early as 72 h after birth with none living longer than 3–4 weeks. The lifespan of the Wwox heterozygotes was indistinguishable from WT mice. Further phenotypic characterization revealed that Wwox ablation resulted in significant growth retardation (dwarfism) that was noticeable at birth. Serum chemistries of Wwox KO mice revealed hypoglycemia, hypocalcemia as well as signs of metabolic acidosis and kidney failure [50,51]. Interestingly, bone histomorphometry showed that Wwox KO mice have severe defects in bone formation in part due to increased osteoclastic activity [50,51]. Much like the hypomorphic Wwox^{gt/gt} mouse, Wwox KO mice were found to have gonadal abnormalities such as testicular hypoplasia in males and reduced ovarian size in females. These effects were suggested to be the result of impaired steroidogenesis [52].

One of the principal aims of ablating Wwox in mouse tissues was to determine whether or not this gene behaves as a classical tumor suppressor. Analysis of spontaneous tumor formation in the two short-lived full Wwox KO models differed. Aqeilan et al. reported spontaneous formation of focal lesions along the diaphysis of Wwox KO mouse limbs

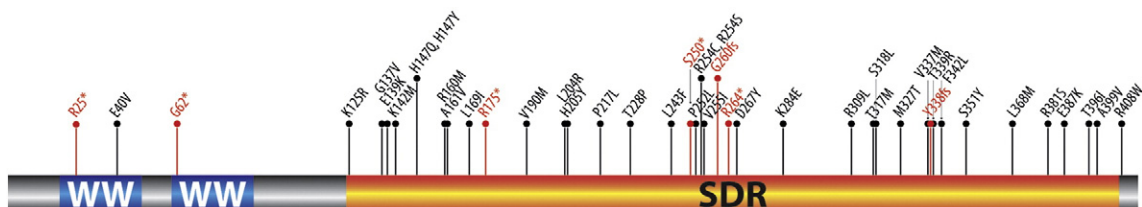


Fig. 7. Novel somatic mutations affecting WWOX in cancer samples. Data obtained from www.cbioportal.org. See Table 1 for detailed description.

Table 1

WVVOX mutations in cancer samples. Data obtained from www.cbioportal.org as per freeze in freeze in February 2014.

Case ID	Cancer study	AA change	Type	Mutation assessor
TCGA-AX-A05Z	Uterine (TCGA) [36]	D267Y	Missense	Low
TCGA-AX-A2H5	Uterine (TCGA) [36]	G260fs	FS ins	
TCGA-AP-A051	Uterine (TCGA) [36]	L169I	Missense	Low
TCGA-AP-A0LM	Uterine (TCGA) [36]	L368M	Missense	Medium
TCGA-BS-A0UL	Uterine (TCGA) [36]	R254S	Missense	Medium
TCGA-BS-A0UF	Uterine (TCGA) [36]	R381S	Missense	Medium
TCGA-AX-A0J1	Uterine (TCGA) [36]	R408W	Missense	Low
TCGA-N7-A4Y0	Uterine CS (TCGA)	L204R	Missense	High
HN_62671	Head & neck (broad) [39]	K142M	Missense	Medium
HN_63080	Head & neck (broad) [39]	R175*	Nonsense	
HN_62814	Head & neck (broad) [39]	V190M	Missense	High
TCGA-CV-7178	Head & neck (TCGA) (in revision)	H147Q	Missense	Neutral
TCGA-CN-4731	Head & neck (TCGA) (in revision)	R25*	Nonsense	
TCGA-CQ-5325	Head & neck (TCGA) (in revision)	S351Y	Missense	High
TCGA-CN-4730	Head & neck (TCGA) (in revision)	Y338fs	FS del	
TCGA-D6-6516	Head & neck (TCGA) (in revision)	R264*	Nonsense	
MEL-Ma-Mel-48	Melanoma (broad) [104]	S318L	Missense	Medium
ME011	Melanoma (broad) [104]	T396I	Missense	Neutral
TCGA-ER-A197	Melanoma (TCGA provisional)	K284E	Missense	Neutral
TCGA-FW-A3R5	Melanoma (TCGA provisional)	L243F	Missense	Medium
TCGA-FW-A3R5	Melanoma (TCGA provisional)	P217L	Missense	Medium
TCGA-EE-A2MN	Melanoma (TCGA provisional)	P252L	Missense	Medium
TCGA-BR-4256	Stomach (TCGA provisional)	F342L	Missense	Low
TCGA-BR-4368	Stomach (TCGA provisional)	M327T	Missense	Neutral
TCGA-CG-5721	Stomach (TCGA provisional)	T317M	Missense	Medium
TCGA-BR-6452	Stomach (TCGA provisional)	V337M	Missense	Low
TCGA-AA-3854	Colorectal (TCGA) [37]	A399V	Missense	Low
TCGA-AG-A02N	Colorectal (TCGA) [37]	K125R	Missense	Low
TCGA-AA-3972	Colorectal (TCGA) [37]	R254C	Missense	High
TCGA-AA-A01Z	Colorectal (TCGA) [37]	T339R	Missense	Neutral
TCGA-AA-A00N	Colorectal (TCGA) [37]	E139K	Missense	Medium
LUAD-74TBW	Lung adeno (Broad) [105]	E40V	Missense	Low
LUAD-B00915	Lung adeno (Broad) [105]	R160M	Missense	Medium
TCGA-55-7576	Lung adeno (TCGA) (in press)	H147Y	Missense	Neutral
TCGA-05-4396	Lung adeno (TCGA) (in press)	G62*	Nonsense	
TCGA-05-4389	Lung adeno (TCGA) (in press)	S250*	Nonsense	
TCGA-60-2720	Lung squ (TCGA) [106]	E387K	Missense	Neutral
TCGA-66-2787	Lung squ (TCGA) [106]	G137V	Missense	High
BR-M-105	Breast (Broad) [107]	G260fs	FS ins	
TCGA-A2-A0CX	Breast (TCGA) [35]	H205Y	Missense	Medium
TCGA-A8-A0A6	Breast (TCGA) [35]	T228P	Missense	Medium
ICGC_0037_TD	Pancreas (ICGC) [108]	V255I	Missense	Neutral
TCGA-AK-3428	Renal clear cell (TCGA) [109]	R309L	Missense	High
TCGA-GV-A3QF	Bladder (TCGA) [110]	A161V	Missense	Low

that appeared neoplastic and were reported as suggestive of either periosteal or chondroid osteosarcoma [50], but this was observed in only 4 pups out of 13 analyzed. In contrast, the analysis of EIIA-Cre; Wwox KO mice found no evidence of spontaneous neoplasia in any tissue examined. This was also the case for EIIA-Cre⁺; Wwox^{+/-fl} heterozygous mice indicating that the loss of one Wwox allele (i.e. haploinsufficiency) appears not to be deleterious or carcinogenic in the longer-lived heterozygous mice [51]. Increase tumorigenicity was reported when Wwox heterozygous mice are exposed to chemical carcinogens [50,53] or when backcrossed into a C3H mammary tumor susceptible genetic background [54].

7.1. Conditional targeted Wwox deletion

Due to the early postnatal death of full Wwox KO mice the phenotypic analysis of Wwox ablation in adult tissues was impossible. To this end conditional, tissue-specific ablation of Wwox was achieved by utilizing Wwox^{fl/fl} mice and crossing them with mice either expressing Cre recombinase under control of the bovine keratin 5 promoter (BK5-Cre) or under the control of the MMTV promoter (MMTV-Cre) for ablation in the mammary gland and other epithelial tissues [55].

Microscopic and morphometric analyses on the effects of Wwox ablation in both conditional deletion models revealed a significant defect in mammary branching morphogenesis in virgin females. However,

this defect did not impede the mammary epithelium from reaching functional maturity during pregnancy as these mice were eventually able to lactate normally. By transcriptome analyses we determined that the expression of the non-canonical Wnt pathway ligand *Wnt5a*, a protein known to inhibit ductal extension and lateral branching [56], was significantly upregulated in Wwox KO mammary epithelium. In addition we observed a significant upregulation and activation of the IL6-Jak-STAT3 pathway and levels of phospho-STAT3 were greatly increased in Wwox KO mammary epithelium from virgin mice [55]. This increase in Jak-STAT3 signaling could be one of the mechanisms by which *Wnt5a* is transcriptionally upregulated [57] and subsequently inhibits ductal morphogenesis. Interestingly however, as will be discussed in following sections, *Wnt5a* is also a direct transcriptional target of the TGF β /SMAD signaling pathway [56].

BK5-Cre; Wwox KO mice were viable, fertile and females were able to lactate. Unexpectedly, these mice died prematurely (68–117 days of age) independent of gender for yet undetermined reasons. In contrast, Wwox ablation via MMTV-Cre had no effect on survival or natural aging. After 1.5 years of follow up of the MMTV-Cre; Wwox KO mice it was determined that the loss of Wwox expression is not carcinogenic in any tissue. Similarly, we did not detect any evidence of malignant development in the shorter-lived BK5-Cre; Wwox KO mice. Furthermore, no evidence of increase proliferation or development of premalignant lesions was observed. Consistent with the findings in the EIIA-Cre

Table 2
Rodent models of WWOX ablation.

Model	Target tissue	Observed phenotypes	Tumors	Reference
Wwox ^{st/st} hypomorph mouse	All tissues	-Moderately shorter lifespan -Reduced fertility -Testicular atrophy	B-cell lymphomas in older female mice	49
Wwox ^{-/-} mouse	All tissues	-Viability 2–3 weeks -Severe dwarfism -Hypoglycemia -Delayed bone formation/osteopenia -Increased osteoclast activity -Gonadal abnormalities -Altered steroidogenesis	Lesions compatible with periosteal osteosarcomas in 4 of 13 KO mice	50,52
EIIA-Cre; Wwox ^{fl/fl} mouse	All tissues	-Viability 3 weeks -Severe dwarfism -Hypocapnia -Leukopenia -Hypoglycemia -Splenic atrophy -Delayed bone formation/osteopenia -Epileptic seizures -Ataxia	No	51
Ide/Ide rat, spontaneous Wwox mutation	All tissues	-Viability 3–12 weeks -Severe dwarfism -Audiogenic epileptic seizures -Low plasma levels of LH and FSH -Leydig cells dysfunction	No	103
BK5-Cre; Wwox ^{fl/fl} mouse	Skin, mammary gland and other <i>Keratin5</i> expressing epithelia	-Viability 10–17 weeks -Impaired mammary branching	No	55
MMTV-Cre; Wwox ^{fl/fl} mouse	Mammary & salivary glands	-Normal lifespan -Impaired mammary branching	No	55
Alb-Cre; Wwox ^{fl/fl} mouse	Liver	-Normal lifespan -Altered lipid metabolism	No	58
Osx-Cre; Wwox ^{fl/fl} mouse	Bone (osteoblasts)	-Normal lifespan -No bone defects	No	Aldaz Lab, unpublished

model, we found that the loss of a single *Wwox* allele (i.e. haploinsufficiency) did not have any observable phenotypic effect in the mammary gland [55].

We further analyzed conditional *Wwox* ablation in additional tissues such as in bone (osteoblasts) using *Osterix-Cre* (*Osx-Cre*) mice and in liver using Albumin-Cre (*Alb-Cre*) mice. Similarly to the described mammary gland models we found no evidence of spontaneous tumor formation in the targeted tissues (unpublished observations and [58]). The observations with the various models of *Wwox* ablation are summarized in Table 2.

8. WWOX in cellular pathways

As earlier mentioned, the WWOX gene encodes a 46 kD cytoplasmic protein containing two functional protein domains, two amino-terminal WW-domains and a carboxy-terminal short chain dehydrogenase domain (SDR) (Fig. 1). The SDR is an enzymatic domain that is predicted to carry out NADP(H)-dependent dehydrogenase reactions with yet to be identified substrates.

We determined that the WWOX protein resides predominantly in the perinuclear region overlapping with the Golgi region [23]. To more precisely define the sequences responsible for perinuclear targeting, we used site directed mutagenesis on GFP-WWOX fusion proteins, to generate large exonic deletions of the SDR domain while keeping the WW domains intact and vice versa deleting the WW domains and preserving the SDR domain. We concluded that proper sub-cellular localization is affected by the disruption of the SDR domain [59]. Furthermore, we generated GFP-WWOX proteins containing single point mutations (S281A, Y293F, and K297A) destroying WWOX's catalytic activity. Remarkably, we observed that the amino acids predicted to be required for WWOX enzymatic activity were also necessary for perinuclear localization (Aldaz laboratory unpublished observations).

In contrast to our observations Chang et al. reported that WWOX localizes to mitochondria and to the nucleus and these investigators speculated that WWOX translocates between these compartments depending on specific stimuli [5,60]. So far we were unable to detect WWOX protein in the nuclear compartment or in mitochondria [23,59] the reasons for this discrepancy remain unclear. A possibility exists however, that WWOX may translocate from different cellular compartments upon very specific signals and at a very rapid rate making difficult its detection in the nucleus or mitochondria.

WW domains are involved in protein–protein interactions with modular proline-rich recognition motifs. The highly compact WW domains (35–45AA in length) are characterized by the presence of a pair of conserved tryptophans (W). These two signature W residues are spaced approximately 20–22 AA apart and play a fundamental role in the structure and function of the domain. WW domains have a very diverse proline-containing sequence preference. Based on such binding preference four groups of WW domains have been described. Two are major and more common, Groups I and II and two less common, Groups III and IV [61]. Group I binds the minimal core consensus Pro-Pro-x-Tyr (PPxY). Examples of WW domain containing proteins from Group I include YAP1, Dystrophin and NEDD4 family members. We and others demonstrated that WWOX WW1 is a Group I WW domain that specifically binds peptides containing the consensus PPxY motif [62,63]. The WWOX WW2 is not a classical WW domain due to the replacement of the second signature tryptophan with a tyrosine at position 85. In a recent report that analyzed the interaction between WWOX WW domains and ERBB4-CTF, it was shown that the WW2 domain does not bind to consensus PPxY motifs but does augment the ability of WW1 to do so [64].

Several proteins containing the PPxY consensus motif have been identified as WWOX interacting partners such as p73, AP2γ, JUN,

EZRIN, ERBB4 (CTF), MET (CTF) and RUNX2. The possible consequences and cancer related implications of these various interactions have been reviewed elsewhere [65]. Several of these proteins are transcription factors and due to the primarily perinuclear localization of WWOX these binding events usually result in the cytoplasmic sequestration and subsequent inhibition of nuclear activity. For instance WWOX has been shown to suppress the transcriptional activity of two p53, homologues, p73 and Δ Np63 α . Inhibition of p73 was reported to result in an increase in apoptosis achieved by sequestering p73 in the cytoplasm [66]. The WWOX interaction with Δ Np63 α has been suggested to result in stabilizing Δ Np63 α by antagonizing the function of the E3 ubiquitin ligase ITCH while also inhibiting Δ Np63 α nuclear translocation and transactivation. It was also reported that the inhibition of Δ Np63 α function by WWOX reverses the resistance of cancer cells to cisplatin [67]. More recently a functional role of WWOX as an inhibitor of the Wnt/beta catenin pathway also due to interaction and cytoplasmic sequestration of Dishevelled family member proteins was suggested [68].

Although the physiological relevance of several of the described *in vivo* interactions requires further research, it is clear that a role for WWOX is emerging as a regulator of transcription by limiting transcription factor access to the nucleus through cytoplasmic sequestration. It is also very likely that these interactions and effects of WWOX on downstream target gene expression may be dependent on cell and tissue context.

8.1. Signaling pathways and biofunctions associated with WWOX

Some authors originally proposed that WWOX behaves as a pro-apoptotic molecule [5]. Furthermore, WWOX overexpression has been reported to induce apoptosis in lung [69], prostate [70] and breast [71] cancer lines among others; in most of these cases the delivery of WWOX was via adenoviral vectors. On the other hand we and others, never observed or reported apoptosis induction in cancer lines from breast, liver, lung or ovarian origin either transiently or stably transfected for WWOX expression [23,72,73]. In agreement with Gourley et al. [72], we hypothesize that WWOX per se is not pro-apoptotic when overexpressed in cancer cells devoid of WWOX expression and the reported pro-apoptotic effect is highly dependent on using delivery vectors that induced significant cellular stress such as adenoviral vectors. Thus the physiological biofunctions and cellular pathways associated with WWOX remain to be better defined.

To identify signaling pathways and biofunctions associated with WWOX expression, we employed the “guilt by association” principle, which states that gene co-expression might indicate shared regulatory mechanisms and roles in related biological processes [74,75]. First, we identified the top WWOX co-expressed genes among 100 breast datasets (approximately 4800 samples, including normal, tumor and breast cancer lines) by using the Multiexperiment Matrix (<http://biit.cs.ut.ee/mem/>) bioinformatics resource. Briefly, we identified the 200 top positively and negatively correlated genes based on the expression profiles of the most reliable WWOX probe sets according to the JetSet (<http://www.cbs.dtu.dk/biotools/jetset/>) scoring approach (219077_s_at and 210695_s_at probes) (Fig. 8A). Second, we used ClueGO [76] and CluePedia Cytoscape’s plug-in [77] for functional analyses, network generation and visualization of the WWOX co-expressed genes. In addition, we employed the Enrichr online resource (<http://amp.pharm.mssm.edu/Enrichr>) for ChIP enrichment analysis (ChEA) [78]. Among the top enriched biofunctions, we found regulation of mammary gland morphogenesis and branching, coenzyme A metabolic process and regulation of cysteine-type endopeptidase activity among other metabolic processes. WNT signaling pathway, senescence/autophagy and fat cell differentiation were also identified as enriched biofunctions associated with the list of WWOX co-expressed genes (Fig. 8B, C). These results are consistent with the previous studies that showed the correlation

between WWOX and ER- α expression in human breast carcinomas [12,13] and the effects of WWOX ablation impairing mouse mammary branching morphogenesis [55]. Importantly and as discussed in the previous sections of this review, recent studies indeed suggested that WWOX is a novel modulator of the canonical and non-canonical WNT signaling pathways [55,68]. In addition, ChIP enrichment analysis allowed us to identify a set of transcription factors that are the most likely to have regulated WWOX associated gene expression changes. We detected a statistically significant enrichment of SOX2 and SMAD transcription factors that are likely responsible for many of the gene sets co-expressed with WWOX. Interestingly and linking the previous *in vivo* [55] and these *in silico* observations, mammary gland branching morphogenesis is intimately link as well with the TGF β /SMAD signaling pathway [79,80]. It is worth noting, as earlier mentioned, that indeed Wnt5a is a transcriptional target of the TGF β /SMAD signaling pathway [56].

8.2. WWOX as a regulator of TGF β signaling

We recently demonstrated that the ablation of WWOX in normal breast cells resulted in significant overexpression of a number of TGF β /SMAD3 target genes known to be essential for cancer progression such as *ANGPTL4*, *PTHLH*, *FST* and *SERPINE1* [81]. We ultimately showed that WWOX acts as an inhibitor of SMAD3 transcriptional activity by sequestering it in the cytoplasm upon TGF β stimulation and inhibiting its occupation of target gene promoters. This inhibition relies upon a WW1 domain-dependent interaction with the SMAD3 ¹⁸¹PPGY¹⁸⁴ motif.

TGF β signaling in breast cells has been an intense and ongoing area of research due to the effects of this signaling pathway being paradoxical. In normal mammary epithelium TGF β signaling acts in a tumor suppressive fashion inhibiting cell growth [79] by activating the expression of cyclin dependent kinase inhibitors such as *CDKN2B* (*p15*) and *CDKN1A* (*p21*) and repressing genes involved in cell proliferation such as *MYC* [82–84]. However, the effect and transcriptional output of TGF β signaling changes dramatically when breast cancers progress to advanced stages, becoming pro-oncogenic and activating subsets of genes involved in cell proliferation, epithelial-to-mesenchymal transition and metastasis [85–87]. The key switches explaining the so-called ‘TGF β paradox’ as tumors progress are still not well understood [88].

We hypothesize that WWOX is a key component contributing to the regulation and homeostasis of the TGF β pathway likely in two critical ways: *Firstly* by binding and preventing the nuclear translocation of receptor-regulated SMADS (R-SMADs) that all contain PPxY motifs (PY box) such as SMAD3 (regardless of their phosphorylation status) and of key co-activators such as RUNX2, thus preventing the transcriptional activation of pro-metastatic genes. *Secondly* by competing with WW domain containing NEDD4 family ubiquitin ligases such as NEDD4L in charge of the degradation of R-SMADs but more importantly also of inhibitory SMAD7 that contains a PY box as well [89]. In yet unreported studies we indeed observed a direct interaction between WWOX and SMAD7. In other words when WWOX is available inhibitory SMAD7 would be protected from degradation, thus serving as a potential reservoir of this important inhibitory protein (Fig. 9).

In summary we propose that the progressive loss of WWOX expression in advanced breast cancer would contribute to deregulating the TGF β pathway and may explain, at least in part, the pro-metastatic effects resulting from TGF β /SMAD3 hyperactive signaling in breast cancer. In support of this hypothesis meta-analysis of three independent breast cancer datasets representing a total of 819 patient samples showed a significant negative correlation between WWOX and *ANGPTL4*. Furthermore, the *WWOX*^{lo}/*ANGPTL4*^{hi} subgroup was enriched in basal-like and triple-negative breast cancer [81].

9. Emerging role of WWOX in other human pathologies

9.1. WWOX in lipid disorders and metabolic syndrome related conditions

Genome wide association studies (GWAS) from various laboratories led to the identification of a region-wide significant association between low levels of high-density lipoprotein cholesterol (HDL-C) and the WWOX gene in dyslipidemic families and low HDL-C cases and controls, as well as in population-based cross sectional and prospective cohorts. Low HDL-C is a well-known major risk factor for coronary artery disease. One particular SNP, rs2548861 in intron 8 of WWOX, demonstrated a very significant association ($p = 6.9 \times 10^{-7}$) with low HDL-C levels [90]. Other studies observed not only an association with HDL-C but also with triglycerides levels as well [91,92]. Very recently resequencing studies by Iatan et al. [58] identified 8 genomic variants significantly associated and segregating with the low-HDL trait in two French Canadian dyslipidemic families. Furthermore in the same study by gene expression profiling we determined that liver specific Wwox conditional KO mice displayed significantly altered lipid metabolic pathways and increased plasma triglyceride levels in female mice, suggesting a significant role for WWOX in modulating HDL and lipid metabolism [58]. The association of the Wwox locus with lipid metabolism is also supported by mouse quantitative trait loci (QTL) maps [93].

Interestingly, multiple studies have implicated the WWOX locus with other metabolic syndrome related conditions such as type 2 diabetes [94,95], hypertension susceptibility [96], coronary artery calcification [97], obesity [98], and left ventricular thickness [99]. Furthermore, by exploring the Phenotype Genotype Integrator resource (PheGenI, <http://www.ncbi.nlm.nih.gov/gap/phegeni>) that merges NHGRI GWAS data with various databases including dbGAP, it is possible to observe an emerging theme of metabolic syndrome related traits significantly associated with SNPs mapping to the WWOX locus including: body mass index, body weight, C-reactive protein levels, insulin levels, cardiovascular disease, cholesterol, blood pressure and various traits associated with cardiovascular parameters among others. Most of



Fig. 11. Tonic-clonic seizures of Wwox knock-out mice. Twenty-day-old Wwox KO mice were exposed to a digital 14 kHz tone. Seizures also develop spontaneously in Wwox KO mice, closely reproducing the human phenotype (101).

these data derives from the NHLBI's Framingham SNP health Association Resource and the Family Heart Study datasets among other sources (Supplementary Table 1).

9.2. Germline loss-of-function WWOX mutations lead to epilepsy, ataxia and mental retardation

In 2007 Gribaa et al. reported a new form of childhood onset autosomal recessive cerebellar ataxia and epilepsy in a large consanguineous family from Saudi Arabia with four siblings affected and mapped its locus to chromosome 16q21–q23 [100], SCAR12, MIM# 614322. Very recently by exome sequencing it was determined that WWOX is homozygously mutated in all four children affected by the described phenotype [101]. The mutation Pro47Thr affects an extremely conserved proline residue that is a critical component of the first WW domain consensus sequence of WWOX. We demonstrated in the same report that

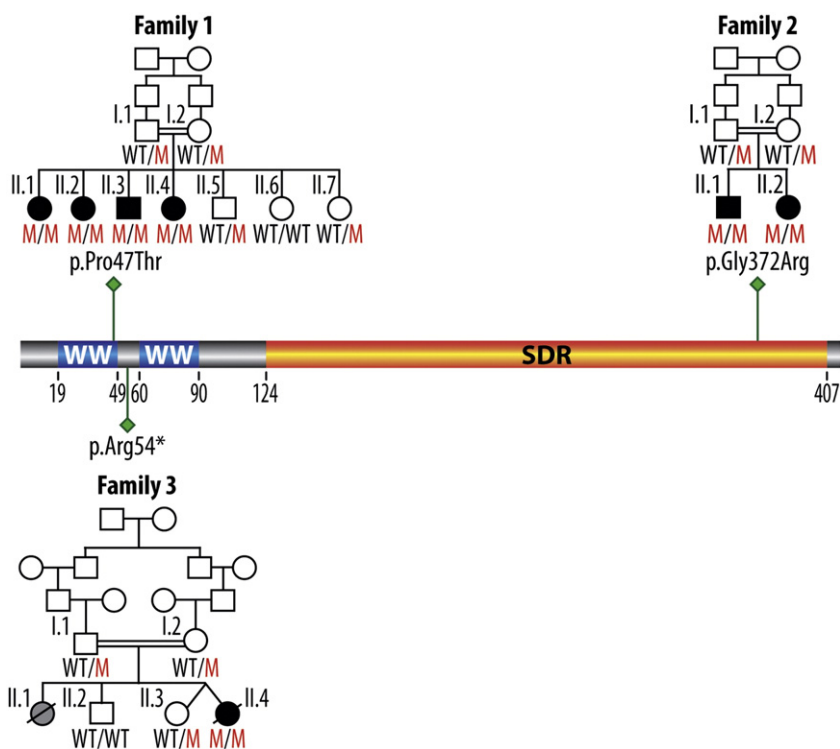


Fig. 10. Germline homozygous WWOX mutations cause severe CNS pathology, predominantly characterized by epilepsy, ataxia and mental retardation. The three human pedigrees and specific homozygous mutations described so far are shown. Families 1 and 2 reported by Mallaret M. et al. (101), family 3 depicting a true full human WWOX knock-out case (p.Arg54*) as reported by Abdel-Salam G et al. (102) characterized by early lethal microcephaly, epilepsy, growth retardation and retinal degeneration.

this mutation renders WWOX unable of binding PPxY target motifs in interacting partner proteins [101]. A second consanguineous family was identified carrying a highly conserved homozygous mutation Gly372Arg of the WWOX protein and the phenotype of the two affected siblings in this family also showed early-onset generalized tonic-clonic epilepsy, mental retardation and ataxia. In this case the mutation affects a glycine found at the C-terminus in the oxidoreductase domain of WWOX and its functional consequences are less clear [101]. Interestingly, an independent report very recently confirmed these observations by describing a new case from another consanguineous family affected by a very similar although more severe phenotype characterized by severe growth retardation, microcephaly, epileptic seizures, retinopathy and early death at 16 months of age. In this case the homozygous WWOX germline alteration is a nonsense mutation at arginine 54 (pArg54*) leading to a virtual WWOX human full knockout case [102]. The homozygous mutations leading to the described human syndromes are summarized in Fig. 10.

Previously Suzuki et al. [103] reported a phenotype reminiscent to that now observed in humans by analyzing the Ide/Ide rats that are characterized by dwarfism, postnatal lethality, male hypogonadism and epilepsy (Table 2). Interestingly, these authors concluded that the Ide mutation is a 13 bp deletion in exon 9 of *Wwox* leading to a frameshift of the last 44 codons of *Wwox* and replacement with a novel open reading frame of 54 codons. These authors could not detect *Wwox* protein by means of Western blot suggesting that this mutation leads to full knockout of the protein.

In the above described report of Mallaret et al. [101] we also demonstrated that *Wwox* full KO mice display an extremely similar phenotype to that observed in humans and rats, since *Wwox* KO mice in their short life-span of 3–4 weeks develop spontaneous and audiogenic epileptic seizures as well (Fig. 11).

In summary, although homozygous mutations affecting WWOX in humans are likely to be rare the described findings underscore the likelihood that WWOX plays a critical role in normal CNS development and physiology.

10. Concluding remarks

In this review we revisited the status of WWOX in cancer and described its emerging role in other important human pathologies. Much still remains to be learned on the physiological role of WWOX, although numerous interacting protein partners have been identified we understand very little on the enzymatic role of WWOX, the true substrate/s of this oxidoreductase remain to be identified.

The mechanistic association of WWOX loss of function with tumor progression warrants further investigation as does the potential role of this protein as modulator of lipid metabolism and its association with metabolic syndrome related conditions.

Finally, the recent identification of the first humans carrying homozygous germline WWOX mutations characterized as suffering early onset epilepsy, ataxia and mental retardation, undoubtedly indicates a key function of this protein in the CNS. The mechanisms on how the loss of WWOX function causes such severe neurological syndromes remain to be defined.

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