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Fhit and Wwox loss-associated genome instability: genome caretaker one-two punch

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Abstract

Expression of Fhit and Wwox protein is frequently lost or reduced in many human cancers. In this report, we provide data that further characterizes the molecular consequences of Fhit loss in the initiation of DNA double-strand breaks (DSBs), and of Wwox loss in altered repair of DSBs. We show that loss of Fhit initiates mild genome instability in early passage mouse kidney cells, confirming that DNA damage associated with Fhit-deficiency is not limited to cancer cells. We also demonstrate that the cause of Fhit-deficient DSBs: thymidine deficiency-induced replication stress, can be resolved with thymidine supplementation in early passage mouse kidney cells before extensive genome instability occurs. As for consequences of Wwox loss in cancer, we show in a small panel of breast cancer cells and mouse embryonic fibroblasts that Wwox expression predicts response to radiation and mitomycin C, all agents that cause DSBs. In addition, loss of Wwox significantly reduced progression free survival in a cohort of ovarian cancer patients treated with platin-based chemotherapies. Finally, stratification of a cohort of squamous lung cancers by Fhit expression reveals that Wwox expression is significantly reduced in the low Fhit-expressing group, suggesting that loss of Fhit is quickly succeeded by loss of Wwox. We propose that Fhit and Wwox loss work synergistically in cancer progression and that DNA damage caused by Fhit could be targeted early in cancer initiation for prevention, while DNA damage caused by Wwox loss could be targeted later in cancer progression, particularly in cancers that develop resistance to genotoxic therapies.

Keywords

Fhit; Wwox; chromosome fragile site; genome caretaker; DNA double-strand breaks

Introduction

FHIT and WWOX are genes that are frequently reduced in expression in many forms of cancer. For several decades scientists have known that the loci in which these genes reside, FRA3B and FRA16D, are among the most fragile in the genome. That is, FHIT and WWOX

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are located at common chromosome fragile sites (CFSs), which are prone to chromosomal breakage on exposure of cells to mild replication stress. As a consequence, the encoded gene products, Fhit and Wwox, are among the most commonly deleted genes across all cancers as noted by two recent large copy number alteration (CNA) studies (Beroukhi et al., 2010; Bignell et al., 2010). The FHIT and WWOX loci exhibit many similarities: they are large genes (1.6 Mb for FHIT, 1.2 Mb for WWOX) straddling CFSs, with mouse knockout (ko) models that show symptoms of increased susceptibility to preneoplasia or neoplasia. Fhit ko mice are more susceptible to cancer than wild-type mice, as they develop more spontaneous and carcinogen-induced tumors (Fong et al., 2000; Zanesi et al., 2001). Wwox ko mice, in which complete ko of Wwox is neonatal lethal, develop spontaneous osteosarcomas in juvenile mice, while haplo-insufficient mice are predisposed to enhanced carcinogen induction of tumors (Aqeilan et al., 2007a, Ludes-Meyers et al., 2007). In addition, re-expression of either Fhit or Wwox in corresponding deficient cell lines reduced cell growth *in vitro* and tumorigenicity *in vivo* (Siprashavili et al., 1997; Ishii et al., 2004; Lewandowska et al., 2009), confirming their tumor suppressor function. Despite these similarities, Fhit and Wwox are entirely dissimilar proteins with distinct structures and functions.

The *FHIT* gene, designated ‘fragile histidine triad’ gene on its discovery, was named for its cytogenetic location spanning FRA3B and for its sequence homology to a superfamily of nucleotide hydrolases and transferases characterized by a conserved histidine triad motif, His-X-His-X-His-xx (where x represents hydrophobic amino acid) (Ohta et al., 1996). Fhit protein was initially shown to function as an Ap_nA hydrolase, cleaving Ap₃A or Ap₄A into adenosine 5'-diphosphate and AMP or ATP and AMP, respectively (Barnes et al., 1996). Recent studies have provided an additional role for Fhit as a scavenger decapping enzyme (Taverniti and Seraphin, 2014), working in collaboration with DcpS to degrade methylguanosine (m⁷Gpppn) 5' cap dinucleotides that are generated by 3' to 5' degradation of mRNA bodies (Li and Kiledijan, 2010). As a tumor suppressor, Fhit has been implicated in caspase-dependent apoptosis, regulation of tumor invasion, epithelial-mesenchymal transition and metastasis (Dumon et al., 2001; Ishii et al., 2001). As a genome caretaker, Fhit maintains the integrity of the genome partially through its regulation of an essential DNA synthesis enzyme, Thymidine Kinase 1 (TK1) (Saldivar et al., 2012; Karras et al., 2016). Loss of Fhit results in down-regulation of TK1 and depletion of nucleotide TTP pools, leading to replication fork stalling and collapse into double strand breaks (DSBs), one of the most detrimental DNA lesions for cells. Importantly, the cellular checkpoint, responsible for pausing the cell to ensure adequate time for DNA repair, is not activated in Fhit-deficient cells, allowing DNA damage to accumulate as the cell continues to replicate. Loss or reductions in Fhit protein expression occur *via* allele deletion or methylation and is one of the earliest genetic or epigenetic alterations of the genome. Therefore, Saldivar et al (2012) proposed that Fhit-deficient cells facilitate clonal expansion of early lesions by supporting checkpoint-blind genome instability that allows selection for oncogene activation and tumor suppressor deletions.

Wwox protein is named for its structural domains and is highly expressed in hormonally-regulated secretory organs, although it is expressed in most organs at lower levels (Nunez et al., 2006; Aqeilan et al., 2007b). WWOX, WW domain containing oxidoreductase, includes two WW domains at its N terminal region and a steroid dehydrogenase/reductase (SDR)-like

region at its center. The SDR domain is likely responsible for the impaired steroidogenesis, hypoglycemia, osteopenia and low HDL-C levels observed in *Wwox* ko mice (Abu-Remaileh & Aqeilan, 2015). The WW domains are protein-binding domains characterized by two conserved tryptophan (W) residues spaced approximately 20 amino acids apart, and interact with other proteins at their PPxY, PPxF, or LPxF motifs (where P is proline, Y is tyrosine, F is phenylalanine, L is leucine, and x is any amino acid). Several proteins have been identified as *Wwox* interactors through the first WW domain: p73, Ap2γ, and ErbB4 (Aqeilan et al., 2004a, 2004b; Schuschardt 2013). Very recently, two studies have delineated roles for *Wwox* in DNA damage response following the formation of DSBs. Abu-Odeh et al. (2014) found that *Wwox* protein was necessary in a signaling cascade that facilitates fulminant activation of ATM, a checkpoint protein that responds to DSBs, while Schrock et al (2016) demonstrated that *Wwox* expression helps dictate what pathway is chosen for repair of DSBs induced by exposure of cells to cytotoxic agents.

We now present data that further characterizes the biological function of FHIT and WWOX genes in DNA damage initiation and response, respectively. We also show in a cohort of squamous lung cancers, that loss of *Fhit* occurs significantly with reductions in *Wwox* protein, and hypothesize that their biological functions work synergistically in the progression of genome instability in cancer cells.

Materials and Methods

Cell lines and reagents

MEFs were isolated from individual 13-day embryos of *Wwox*^{+/+} and *Wwox*^{-/-} mixed background (B6 × 129 SvJ) strain pregnant females and designated MEF WT4, WT7, KO3 and KO5 cell lines. They were cultured in DMEM with 10% FBS, 100 µg/ml gentamicin and established as described (Miuma et al., 2013). Breast cancer cell lines, MDA MB-231, ZR-75-1, BT-20, T47D, MDA MB-453, and MDA MB-436, previously examined for numerous cancer associated expression changes (Neve et al, 2006; Shibata et al, 2011), were maintained as described (Shibata et al., 2011).

Establishment of mouse kidney cell lines were described previously (Karras et al., 2016; Miuma et al., 2013). Briefly, minced mouse kidney tissue from *Fhit*^{+/+} C57Bl6 and *Fhit*^{-/-} (B6×129SvJ backcross) 5-week-old mice were cultured and epithelial cells emerged to fill the culture vessel. Protein was collected at alternate passages. To establish nutritionally stressed (NS) cell lines, early passage cells were maintained without replenishing medium for several months, followed by fresh medium and subculturing of surviving colonies; +/+ cell lines did not survive nutritional stress. The kidney cell lines were cultured in MEM with 5% FBS and 100 µg/ml gentamicin.

Comet Assay

Neutral comet assays were performed using the CometAssay kit (Trevigen) and recommended protocol. Images were acquired with a Zeiss Axioscop 40 fluorescent microscope mounted with an AxioCam HRc camera, and using an A-Plan 10×/0.25

objective lens. Images were converted to Bitmap files using Axiovision 3.1 software, and comet tail moments were scored using Comet Score 1.5 (TriTek, autocomet.com).

Immunofluorescence

Cells were grown on 8-chamber slides (Lab-Tek II) and fixed in freshly prepared 4% paraformaldehyde (PFA). Samples were rinsed with PBS, permeabilized in 0.1% TritonX-100 and blocked in 1% BSA. Cells were incubated with primary antisera, mouse anti- γ H2AX, at 1:333 dilution (Millipore) overnight at 4°C. Slides were washed 3×10 min in PBS, and secondary antiserum (AlexaFluor 488 – conjugated donkey anti-mouse IgG, 1:280, Invitrogen Molecular Probes) was added and incubated for 1 h at room temperature. Slides were washed and coverslips mounted using Fluoro-Gel II – with Dapi (Electron Microscope Sciences). Images were acquired at room temperature with an Olympus FV1000 spectral confocal microscope, a UPLFLN 40XO objective lens, NA 1.30, and with Olympus FLOWVIEW acquisition software.

Western Blot

Whole cell lysates were prepared in RIPA buffer (Thermo Scientific) supplemented with Halt Protease cocktail Inhibitors (Thermo Scientific). Proteins were separated by SDS gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted with antisera against mouse Fhit (Fong et al., 2000), GAPDH (Calbiochem), Vinculin (AbCam), and TK1 (Proteintech).

Stratification of large cancer cohorts by Fhit expression: identification of shared cancer driver pathways in Fhit negative cancers

354 lung tumor samples (Affymetrix data) were stratified by level of Fhit expression and then examined for levels of expression for IMPDH2, IMPDH1, DCK, PPAT, NME1, TK1, RRM2, TYMS, and PRPS1 genes.

Clonogenicity

Clonogenicity assays were performed as described (Miuma et al., 2013). Cells were harvested immediately after exposure to IR and 4 hrs following exposure to MMC, 24 hrs after ABT-888, and 2 hrs after bleomycin treatment. Survival (%) was calculated based on plating efficiencies of cells with no exposure to treatment.

KM Plotter and Gene expression using publicly available databases

For data in Fig. 5A, the Kaplan Meier Plotter was accessed <http://kmplot.com/analysis/index.php?p=background> and patients receiving platinum-based chemotherapy were selected from the ovarian cancer patient cohort and divided into two groups (high and low WWOX expression) according to the quantile expression of Affymetrix 210695_s probe corresponding to the WWOX gene. Patient progression-free survivals were then plotted for the two groups.

To compare the expression of Wwox in Fhit-normal and Fhit-low cancers (Fig. 5B), lung squamous cell carcinoma data was downloaded through TCGA. Fhit and Wwox were

normalized to GAPDH in Normal and tumor datasets. Tumor values were then divided by normal to determine reductions in Fhit and Wwox.

Statistical analysis

Nonparametric data was analyzed using the Mann-Whitney rank sum test for single comparisons or using the Kruskal-Wallis test for multiple comparisons. P-value <0.05 was considered statistically significant.

Results and Discussion

Thymidine supplementation prevents ongoing DNA damage in Fhit^{-/-} cells

Fhit knockout mouse kidney cells exhibit a dramatic reduction in thymidine kinase (TK1) protein expression compared to $+/+$ cells (Fig. 1A). This observation that TK1 protein expression changes based on Fhit status has previously been demonstrated; Fhit silencing in HEK293 and A549 lung adenocarcinoma cell lines resulted in decreased TK1 expression, and induction of Fhit expression in H1299 lung carcinoma cells restored TK1 protein expression (Saldivar et al., 2012; Kiss et al., 2016). Downregulation of TK1, due to loss of Fhit, causes dNTP imbalance, resulting in spontaneous replication stress that leads to chromosomal aberrations, allele copy number variations, small insertions/deletions and single-base substitutions (Saldivar et al., 2012; Miuma et al., 2013; Paisie et al., 2016). Therefore, to confirm the role of the Fhit-TK1 pathway in promoting genome stability, we asked if Fhit-deficient cells exhibit decreased levels of DNA damage upon addition of a continuous supply of thymidine, the substrate for TK1, despite the low TK1 protein expression of $-/-$ cells (Fig. 1A). We first assessed spontaneous levels of damage by quantifying nuclear γ H2AX foci, marker of DSBs, by indirect immunofluorescence in early passage $+/+$ and $-/-$ kidney cell lines (Fig. 1B). The $-/-$ cells exhibited ~2-fold increases in γ H2AX positive foci vs $+/+$ cells (Fig. 1C). Levels of DNA damage prior to thymidine supplementation were also measured in these cells by neutral comet assay, a method that relies on the principle that fragmented DNA from DSBs migrates faster than intact DNA through a gel when subjected to an electrical current. Subsequent staining with a DNA intercalating agent is used to visualize the nucleoid, seen as a comet head representing undamaged DNA, and the budding “comet tail” that represents the fragmented DNA that migrated out of the nucleoid. DSB levels of individual cells were quantified by measuring tail moment, a measure that combines the amount of DNA within the tail as well as the distance of migration. We observed a significant increase in the mean tail moment of Fhit $-/-$ vs $+/+$ cells, indicating that $-/-$ cells exhibit elevated levels of damage (Fig. 1D,E). These results are in accord with previous studies demonstrating that loss of Fhit expression causes spontaneous DNA damage (Saldivar et al., 2012). Low level concentration (10 μ M) thymidine supplementation for 40 days suppressed DSB formation in $-/-$ cells only (Fig. 1F), with supplemented cells showing mean tail moments similar to $+/+$ cells, demonstrating that $-/-$ cells do not accumulate further damage upon addition of thymidine.

TK1 reactivation in Fhit-negative cancer cells

Intriguingly, as we followed the lifespan of mouse kidney cells *in vitro*, we noted that late-passage $-/-$ and NS3 kidney cells show restored levels of TK1 protein (Fig. 2A). NS3 is an

in vitro transformed kidney cell line. These cells express a mutant p53 that disrupts G1 cell cycle arrest, display an EMT phenotype, and exhibit invasive properties (Karras et al., 2016). Therefore we asked if thymidine supplementation could prevent ongoing genome instability in cells that exhibit an increased mutation burden and signal pathway alterations. Levels of DNA damage were again assessed by a neutral comet assay. The addition of thymidine to NS3 cells did not inhibit the accumulation of damage as observed for early passage $-/-$ cells (Fig. 2B), suggesting NS3 cells may have accumulated other genetic/epigenetic alterations that contribute to genome instability. Interestingly, TK1 mRNA expression is frequently up-regulated in cancers, leading to TK1 over-expression. Expression analysis of 354 lung tumor samples obtained from The Cancer Genome Atlas revealed a negative correlation between Fhit and TK1 (correlation coefficient = -0.251) (Fig. 2C) (S. Volinia, personal communication). Over-expression of other DNA synthesis enzymes, TYMS and RRM2, observed in this same expression profile, suggests that there is selection in cancer cells for increased dTTP production. It would be advantageous for cancer cells to select for these alterations to maintain a highly proliferative status. Collectively, these results suggest that TK1 down-regulation by Fhit loss is a transient step initiating genome instability in preneoplastic lesions. Since most of these lung cancers are negative for Fhit protein expression, the recovery of TK1 expression is independent of Fhit expression, indicating that cancer cells find novel ways to bypass the modulation of TK1 expression by Fhit, possibly through increased TK1 protein stability.

Proposed model for Fhit regulation of TK1 through scavenger decapping

Since dTTP pool imbalance caused by loss of Fhit/TK1 facilitates the progression of cancer through induction of replication stress and subsequent genome instability, it is important to understand the mechanism by which Fhit regulates TK1. A recent study reporting a novel role for Fhit in metabolizing free mRNA cap (m7GpppN) dinucleotides that are generated by 3' to 5' mRNA degradation, suggests a molecular mechanism through which Fhit might modulate the expression level of TK1, and other downstream Fhit targets, through affecting the translation of a specific cohort of mRNAs (Taverniti and Seraphin, 2015). Interestingly, the structure of 5' mRNA caps closely resembles that of dinucleoside triphosphates, a favored *in vitro* substrate of Fhit, ie, molecules which Fhit has been shown to hydrolyze *in vitro* (Fig. 3A) (Barnes et al., 1996). Additionally, cancer cell lines that have lost Fhit expression produce excess Ap3A (Murphy et al., 2000). So how does hydrolysis of free m7GpppN cap structures by Fhit affect TK1 expression? Eukaryotic cap structures are co-transcriptionally added to the 5' terminus of mRNAs and serve to protect nascent transcripts from degradation and assist in ribosome binding to initiate translation. Thus, regulation of gene expression is tightly regulated through mRNA decay, in which two main pathways exist. Removal of the poly(A) tail is the first step in both pathways. Deadenylation is followed by removal of the cap and subsequent digestion of the mRNA body by a 5' exonuclease in the 5' to 3' decay pathway. Alternatively, the mRNA body can be digested by the exosome in the 3' to 5' direction resulting in the release of free cap dinucleotide structures. Scavenger decapping enzymes can then bind and hydrolyze these free cap (m7GpppN) dinucleotides. Accumulation of free caps has been shown to have deleterious effects and impact pathological conditions (Gogliotti et al., 2013). Thus, scavenger decapping enzymes play a pivotal role in clearing free cap dinucleotides from the cell to

maintain steady-state levels of translating mRNAs. We hypothesize that following Fhit loss, a decrease in scavenger decapping activity would result in aberrant accumulation of m⁷GpppN dinucleotides that might compete with and sequester translation initiation factors from selected capped mRNA substrates. This proposed model suggests a mechanism for TK1 down-regulation observed following Fhit loss and a path to identification of other Fhit downstream targets that may participate in the genome caretaker and tumor suppressor activity of the Fhit protein, as outlined previously (Kiss et al., 2016).

Wwox-deficient cells exhibit increased cell survival on exposure to various genotoxic agents

DSBs, acquired endogenously or exogenously, can be repaired through four different pathways. Non-homologous end-joining (NHEJ) briefly processes the ends and religates them independent of sequence homology. Homology Directed Repair (HDR) requires 5' end single strand DNA resection and the presence of a homologous sequence (usually on a sister chromatid) to serve as template for new DNA synthesis. Both Single Strand Annealing (SSA) and Alternative NHEJ (Alt-NHEJ) involve more extensive DNA resection up to a region of microhomology on both DNA strands, but have different resolutions for rejoining DNA. Recently, our lab discovered that Wwox expression modulates the efficiencies of all four DSB pathways in normal and cancer cells exposed to ionizing radiation (IR), such that absence of Wwox protein expression, commonly seen in cancerous cells, leads to enhanced HDR and SSA repair, but decreased NHEJ and Alt-NHEJ repair (Schrock et al., 2016), in comparison to results of Wwox-expressing cells. These alterations in DSB repair enable Wwox-deficient cancer cells to survive DSB-inducing agents significantly better than Wwox-expressing cells, an adverse patient response to a number of therapeutic chemotherapies. To confirm the role of Wwox in survival of exposure to such agents, we employed cancer cell lines that were neither induced nor silenced for Wwox expression and performed clonogenicity assays on a small panel of human breast cancer cell lines: ZR-75-1, BT-20, T47D, MDA MB-453 and MDA MB-436 cells following increasing IR doses, which induce primarily DSBs (Fig. 4A). As expected, in the percentage of cells that survived radiation, we observed a correlation of the level of endogenous Wwox expression with decreased survival. That is, ZR-75-1 and BT-20 cells express normal levels of Wwox and exhibit ~10-fold lower survival at 4 Gy compared to T47D, MDA MB-453 and MDA MB-436 cell lines, which were Wwox-deficient. We also determined cell survival in an epithelial mouse cell line, kd2, derived from kidney, at early passage (p 10) and at late passage (p 23) where Wwox protein was reduced (Fig. 4B). This cell line at late passage (Wwox-reduced) exhibits a significant and striking survival compared to the early passage at 4 Gy ($P < 0.0001$). Next, we investigated the effect of Wwox expression on sensitivity to the crosslinking chemotherapeutic agent Mitomycin C (MMC) and the combination treatment of MMC + ABT-888, PARP inhibitor. We hypothesized that Wwox-deficient cells would be resistant to these agents because they cause DNA lesions, which ultimately lead to DSBs, and loss of Wwox results in enhanced HDR repair of DSBs. Indeed, ko MEFs exhibited enhanced survival to 4 hr MMC exposure at concentrations 1 μ M and higher ($P < 0.01$), and an even greater resistance noted after MMC treatment combined with ABT-888, an inhibitor of PARP1 activity ($P < 0.001$) (Fig. 4C, 4D). Collectively, this data confirms that Wwox loss

supports resistance to DSB-inducing agents and that Wwox-deficiency provides a survival advantage to cancer cells carrying DSBs

Patients with Wwox-deficient cancers have poorer outcomes of chemotherapeutic treatments

Since reduction of Wwox protein enables normal and cancer cells to resist genotoxic treatments *in vitro* (Schrock et al., 2016), we predicted that patients with Wwox-deficient tumors would be more resistant to DSB-inducing chemotherapies, resulting in more rapid/frequent cancer recurrence and decreased patient survival. To further explore the prognostic value of Wwox expression in cancers, we employed the Kaplan-Meier Plotter resource (Gyorffy et al., 2013) to evaluate the effects of Wwox expression on patients receiving DSB-inducing agents for cancer treatment. Kaplan-Meier analysis revealed that ovarian cancer patients with Wwox-deficient cancers that received Platinum-based chemotherapy exhibited shorter progression-free survival ($n = 1185$) compared to patients with Wwox-expressing cancers (Fig. 5A). This data strongly supports our hypothesis that dysregulation of DSB repair in Wwox-deficient cells facilitates cancer cell survival to treatment and worsens patient outcomes.

Loss of Fhit occurs frequently with loss of Wwox

Given the biological functions of Fhit and Wwox, we hypothesize that coordinate loss of both genes would enhance genome instability because Fhit and Wwox-deficient cells would exhibit more DSBs that are subject to dysregulated DSB repair. Therefore, we became interested in identifying cancers which have simultaneous loss of Fhit and Wwox protein. Our lab has previously shown that Fhit-deficient mouse kidney cells exhibit a two-fold increase in chromosomal breaks at CFSs compared to Fhit-sufficient mouse kidney cells (Hosseini et al, 2013). The most frequently altered CFS in Fhit-deficient mouse and human cells corresponded to the Wwox locus, suggesting that activation of Wwox quickly succeeds loss of Fhit. This has been demonstrated in a subset of invasive breast carcinoma patients ($n=97$) where Guler et al. (2004) revealed through immunohistochemistry that 55% of patients had reduced Fhit expression, 63% had reduced Wwox expression, and the loss of one gene significantly correlated with the loss of the other ($P=0.001$) and with metastatic tumors ($P<0.05$), suggesting perhaps that their simultaneous loss associates with more aggressive cancers. To determine if this is true in other cancer types, we accessed The Cancer Genome Atlas and identified a dataset of squamous lung cancers. First, Fhit and Wwox expression was normalized by GAPDH expression, then tumors were ranked by Fhit expression into Fhit normal and Fhit low groups. Fig. 5B demonstrates that the average expression for Wwox was significantly decreased ($P<0.05$) in the Fhit-low group compared to the Fhit-normal group, suggesting loss of Fhit is accompanied by reduction in expression of Wwox.

Conclusions

In this study we have further defined functions of Fhit and Wwox. We show that Fhit regulates dTTP levels and suggest that this occurs through scavenger decapping of TK1 mRNA in accord with the Taverniti and Seraphin report of 2015; we also showed that Wwox

alters survival of exposure to genotoxic agents, presumably through dysregulation of DSB repair. We hypothesize that combined loss of these genes would greatly increase genome instability in a cancer cell and be more detrimental to patients than their singular loss. In support of this, a subset of cancer patients with invasive breast carcinomas have combined loss of Fhit and Wwox in approximately 50% of samples with a significant correlation to more aggressive, metastatic disease (Guler et al, 2004).

Loss of Fhit protein is known to be one of the earliest events in cancer development (Gorgoulis et al., 2005; Bartkova et al., 2005) and our lab has proposed this as an initiating event in cancer whereby low levels of DNA damage (DSBs) are undetected by cell cycle checkpoints and provide an environment primed for selection of mutations advantageous to cancer cells (Saldivar et al., 2012; Miuma et al., 2013). On the other hand, loss of Wwox promotes cell survival of exposure to DSB-inducing agents used in cancer treatment, implying that the Wwox-deficiency phenotype may be particularly important during cancer progression and in resistance to genotoxic treatment. Therefore, we propose that intervention in Fhit-deficient early lesions should be considered as a prevention strategy for preneoplasias, and the Wwox-deficient condition should be considered in conjunction with DSB-inducing treatment regimens. More research is needed to develop prevention strategies and to define in detail the effects of combined Fhit and Wwox loss on genome instability and cancer progression.

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Fig. 1. Thymidine supplementation prevents ongoing DNA damage.

A) Immunoblot of Vinculin and TK1 in $-/-$ and $+/+$ mouse kidney cells. **B)** Indirect immunofluorescence of γ H2AX, before thymidine supplementation. **C)** Quantification of γ H2AX-positive cells before thymidine supplementation. Bar graph indicates the means, and error bars represent the standard error. **D)** Neutral comet assay of mouse kidney cells before thymidine supplementation. Box plots of tail moments include data (WT, $n = 285$; $Fhit^{-/-}$, $n = 435$) from 3 separate experiments. **E)** Representative photos of $Fhit^{-/-}$ and $Fhit^{+/+}$ comet tails. **F)** Neutral comet assay of mouse kidney cells 40 days post 10 μ M thymidine supplementation. Box plots of tail moments include data (WT untreated, $n = 344$; WT with thymidine, $n = 228$; $Fhit^{-/-}$ untreated, $n = 341$; $Fhit^{-/-}$ with thymidine, $n = 286$) from 3 separate experiments. dT, thymidine.

Fig. 2. TK1 reactivation in Fhit-negative cancer cells.

A) Western blot analysis of TK1 expression in mouse kidney cells. In +/+ cells, TK1 expression decreases in parallel to loss of Fhit expression. In -/- cells, loss of Fhit results in transient TK1 downregulation, as TK1 expression increases at late passage. **B)** Assessment of DNA damage *via* neutral comet assay in mouse kidney cells. Fhit-/- NS3 is a nutritionally stressed cell line that has undergone cellular transformation *in vitro* and over-expresses TK1. Box plots of tail moments include: Fhit+/+ without thymidine, n=72; Fhit +/+ with thymidine, n=103; Fhit-/- without thymidine, n=282; Fhit-/- with thymidine, n=331; NS3 without thymidine, n=144; NS3 with thymidine, n=161. Thymidine supplementation (10μM) for 16 days and includes data from two separate experiments. No significant difference in levels of damage between mock and dT of Fhit+/+ (p=0.4095) and Fhit-/- NS3 (p=0.5729). dT, thymidine. **C)** RNA-seq data obtained from The Cancer Genome Atlas shows a negative correlation between Fhit expression and expression of enzymes involved in dTTP synthesis (TK1, RRM2, and TYMS). Red, up-regulated. Green, down-regulated. We conclude that during cancer progression, there is selective pressure to increase expression of these proteins needed for balanced dNTP pools and for optimal DNA replication.

A Diadenosine triphosphate

B

Fig. 3. Model for Fhit as a scavenger decapping enzyme regulating translation of TK1 mRNA.

A) Structure of diadenosine triphosphate (Ap3A), the first recognized *in vitro* substrate for Fhit, and the 5' 7-methyl-guanosine cap. **B)** In the 3'' to 5'' mRNA decay pathway, the exosome generates free m7GpppN dinucleotides that can be hydrolyzed by a scavenger decapping enzyme. The model hypothesizes that In the presence of Fhit, Fhit binds and hydrolyzes m7GpppN into m7GDP and m7GMP, which are cleared from the cell. In the absence of Fhit, free m7GpppN caps accumulate. Preferential binding of translation initiation factors to these free caps instead of capped TK1 mRNAs leads to deregulated translation of TK1 mRNA. As the model proposes, Fhit is thus a scavenger-decapping enzyme that eliminates residual cap structures to promote ribosomal binding and translation of cap-bearing mRNAs.

Fig. 4. Wwox-deficient cells are significantly more resistant to various genotoxic agents.

A) Graph of % survival of a small panel of five breast cancer cell lines following exposure to various doses of gamma IR. **B)** Graph of % survival of mouse epithelial cell line, kd2, at early and late passage following various doses of gamma IR. **C)** Line graph depicting % survival of Wwox MEFs following exposure to MMC for 4 hrs. **D)** Line graph depicting % survival of Wwox MEFs following exposure to MMC for 4 hours + ABT-888 for 24 hours. Embedded graph depicts cell survival of MEFs with ABT-888 treatment alone. **(A-B)** Embedded western blots depict Wwox expression in the cell lines. **(A-D)** Error bars depict standard error and are the results of three independent experiments.

Fig. 5. Fhit and Wwox expression affect cancer patient survival.

A) Kaplan-Meier plot of ovarian cancers treated with Platinum-based agents and separated into Wwox-reduced or Wwox normal/high groups. Patients with Wwox-deficient cancers have significantly shorter progression-free survivals ($P < 0.001$, $n=1185$). **B)** Box plot depicting relative Wwox expression in lung squamous cell carcinomas categorized into two groups: Fhit normal and Fhit low.