Fhit Modulates the DNA Damage Checkpoint Response

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Abstract

In preneoplastic lesions, the DNA damage checkpoint is induced and loss of heterozygosity at the FRA3B/FHIT common chromosome fragile region precedes or is coincident with activation of the checkpoint response in these early stages. Introduction of exogenous Fhit into cells in vitro led to modulation of expression of checkpoint proteins Hus1 and Chk1 at mid-S checkpoint, a modulation that led to induction of apoptosis in esophageal cancer cells but not in noncancerous primary cultures. Mutation of the conserved Fhit tyrosine 114 resulted in failure of this function, confirming the importance of this residue. The results suggest that the DNA damage–sensitive FRA3B/FHIT chromosome fragile region, paradoxically, encodes a protein that is necessary for protecting cells from accumulation of DNA damage through its role in modulation of checkpoint proteins, and inactivation of Fhit contributes to accumulation of abnormal checkpoint phenotypes in cancer development. (Cancer Res 2006; 66(23): 11287-92)

Introduction

During early stages of cancer development, precancerous cells experience selective pressure to escape from cell cycle block induced by checkpoint responses to DNA damage. To overcome the block, DNA damage checkpoint genes are frequently mutated during cancer development. Indeed, recent studies of lung and skin hyperplasias and dysplasias (1, 2) showed that from early dysplastic stages, an Atr/Atm–regulated DNA damage response network is activated, delaying or preventing cancer. Mutations compromising this checkpoint, including defects in the Atm-Chk2 and Atr-Chk1 pathways, would allow cell proliferation, survival, and tumor progression, leading to further DNA replication stress, increased genomic instability, and pressure for accumulating mutations (1, 2).

Common fragile site lesions exhibit gaps or breaks in metaphase chromosomes when cells are exposed to replicative stress, and FRA3B and FRA16D, at chromosomes 3p14.2 and 16q23.3, are the two most active of the common human fragile sites (3). Previous studies have shown involvement of repair-associated proteins in control of fragile site integrity, including Atr (4), Brca1 (5), Smc1 (6), and Fanconi anemia pathway proteins (7). A recent study showed that homologous recombination and nonhomologous end-joining repair pathways regulate FRA3B and FRA16D fragile site stability, indicating that double-strand breaks are formed at common fragile sites due to replication perturbation (8).

The 1.7 MB FHIT gene spans FRA3B, encodes a 1.2-kb mRNA and a 16.8-kDa protein, and is frequently involved in biallelic loss and other chromosome abnormalities in tumors (9). FHIT deletions, abnormal transcripts, promoter hypermethylation, and associated loss of Fhit expression are common in human malignancies (10–12). Indeed, loss of FHIT alleles is among the earliest alterations observed in preneoplastic areas of carcinogen-exposed lung tissues and loss of Fhit expression is observed in premalignant lesions of esophagus, stomach, cervix, and other organs, suggesting that loss of Fhit expression, due to the susceptibility of FHIT/ FRA3B to carcinogen damage, plays a role in initial stages of multistep carcinogenesis (11).

Fhit overexpression experiments following plasmid, adenoviral, or adeno-associated virus gene transfer into cancer cell lines have shown that Fhit suppresses cell growth and induces caspase-dependent apoptosis in cancers and cancer-derived cell lines (13–17) but has little effect on noncancerous cells (14, 18). Fhit+/− and Fhit−/− mice exhibit increased susceptibility to spontaneous and carcinogen-induced tumors (19–21) and viral-mediated Fhit expression prevented development of induced tumors (22, 23). The animal experiments suggested a selective Fhit suppressive effect on abnormally proliferating precancerous and cancerous lesions.

Fhit can be phosphorylated at amino acid Y114 by Src tyrosine kinase family proteins (24). Biochemical analyses indicated that the ApA hydrolase enzyme kinetics of recombinant Fhit, monophospho-Fhit, and diphospho-Fhit differed in steady-state KM and kcat values, with KM Values for monophospho-Fhit and diphospho-Fhit lower than for Fhit (25). Because Fhit function is determined by binding of substrate, influenced mainly by low KM, the Y114 site should influence suppressor function. Thus, we examined the effects on apoptosis, DNA damage response, and checkpoint proteins, following introduction of FHIT and FHTY114 mutants into normal and cancer cells. Introduction of wild-type FHIT (FHIT-Wt), unlike with the mutants, led to modulation of expression of checkpoint proteins and sensitized cancer cells to induction of apoptosis, suggesting a role in surveillance of genome integrity. Thus, the FHIT gene, perhaps the very first target of induced DNA damage, encodes a protein that is necessary for protecting cells from accumulation of this type of damage through its role in modulating checkpoint responses.

Materials and Methods

Cell culture. Human embryonic kidney cells and esophageal cancer cells (26) were cultured in DMEM with 10% fetal bovine serum (10% DMEM). Primary human fibroblasts (PFB; Cambrex, East Rutherford, NJ) were used.
within two passages. For synchronization, cells were cultured in medium with 0.05% serum for 24 hours and released into S phase by culturing in 10% DMEM. Viable cells were defined as those excluding trypan blue, as visualized by vital staining, and with G1 or greater DNA content. For UV irradiation, 60% to 70% confluent monolayer cells were irradiated with UVC emitted by a germicidal lamp (GL-15; NIPPO, Tokyo, Japan) emitting predominantly 254 nm.

**Plasmids, siRNAs, and viruses.** The plasmids pBJF-FLAG-ATRwt and pBJF-FLAG-ATRkd (provided by S. Schreiber and K. Cimprich) were cotransfected with a G418-resistant pDNA3 vector (BD Biosciences, San Jose, CA) at a molecular ratio of 50 (ATR plasmids) to 1 (G418 plasmid) using calcium phosphate. For selection, cells were cultured in 10% DMEM with G418 (200 μg/mL) for 2 to 3 weeks. Oligo siRNAs for FHIT, RAD1, and luciferase were synthesized (TAKARA, Mie, Japan) and transfected using TransIT-TKO (Mirus, Madison, WI). Cells were transfected with 10 nmol/L annealed oligo siRNA using 4-μL reagents in 100-μL DMEM and cells were analyzed after 24 hours. Construction and purification of the viruses were previously described (27).

**Protein analysis.** Cells were lysed in 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 2% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na3VO4, 1 μg/mL leupeptin, separated by 4% to 20% SDS-PAGE, transferred to membrane, and probed with primary antisera. Primary antisera used were anti-Hus1, Chk1, Rad9, Atr (sc-1887; from Santa Cruz Biotechnology, Santa Cruz, CA), Orc2, Grb2 (from BD Biosciences), Atr (ab-2, EMD Biosciences, San Diego, CA), pChk1(Ser317), caspase-7 (from Cell Signaling, Danvers, MA), phospho-H2AX (Upstate, Lake Placid, NY), Cdc25A (LabVision, Fremont, CA), Flag (Sigma, St. Louis, MO), and actin (ICN, Irvine, CA), and were detected with secondary antisera in the enhanced chemiluminescence system (Amersham, Tokyo, Japan). For immunoprecipitation, 50-μg protein, precleared with protein G-Sepharose beads, was incubated with antiseraum (3 μg) overnight, immobilized on protein G-Sepharose beads, and washed five times in lysis buffer, eluted by boiling, and subjected to SDS-PAGE and immunoblotting. MG-132 proteasome inhibitor was added to cells (50 μM/mL), then cells were incubated for 6 hours and lysed in buffer containing 5 mmol/L N-ethylmaleimide.

**Immunohistochemistry.** Murine forestomach tissue sections from previous experiments (23) were examined for expression of specific proteins in carcinogen-induced lesions of mice that received oral adeno-associated FHIT virus. After antigen retrieval, endogenous peroxidase was inhibited with 3% hydrogen peroxide and nonspecific binding sites were blocked with normal goat serum. Slides were incubated with primary antisera against FHIT (Zymed, Carlsbad, CA), Bax (2772, Cell Signaling; N-20, Santa Cruz Biotechnology), Hus1 (Santa Cruz Biotechnology), or pChk1(Ser317) (Cell Signaling), followed by incubation with biotinylated secondary antisera and detection with streptavidin horseradish peroxidase (Dako, Glostrup, Denmark; 1:10,000).

### Results and Discussion

**Role of Fhit in apoptosis induction.** We assessed the effect of wild-type and mutant Fhit protein expression after infection of a panel of esophageal cancer-derived cells with adFHIT-Wt or adFHIT-Y114F (alanine) or adFHIT-Y114F (phenylalanine) mutant viruses. Immunoblot analysis of proteins in infected cell lysates indicated that Fhit, Fhit mutants, and control green fluorescent protein (GFP; not shown) were successfully expressed in normal fibroblasts, as well as cancer cells (Fig. L1 and Fig. S1A). Previous experiments indicated that effects of Fhit are apparent 48 to 72 hours after introduction of Fhit (14, 16); thus, most experiments were done at 72 hours. Flow cytometric analysis showed efficient induction of a sub-G1 fraction, representing apoptotic cells, at 72 hours after infection with FHIT-Wt, but not with mutant FHIT nor GFP, in TE14 esophageal cancer cells, whereas normal PFBs were unaffected by Fhit overexpression unless UV exposed (Fig. LB); exposure of TE14 cells to UV resulted in enhancement of apoptosis induction by FHIT-Wt infection and induction of a small sub-G1 fraction by FHIT-Y114F infection. Figure 1B and Fig. S1 show that, among the cell types examined, there were two different responses to FHIT and mutant viruses after viral infection or infection followed by UV exposure: (a) TE14 and TE4.1 cells exhibited large sub-G1 fractions after infection with FHIT-Wt virus but not after mutant infections (Fig. LB and Fig. S1B); (b) TE1, TE12, and TE13 cells were not affected at 72 hours after infection, unless subsequently exposed to UV; UV causes these cells to develop sizable sub-G1 fractions after FHIT-Y114F virus infection and smaller sub-G1 fractions in some TE cells by mutant viruses, particularly Y114F virus, suggesting that this mutant retains some Fhit function (Fig. S1B); similarly, PFBs show no induction of a sub-G1 fraction after infection with Wt or mutant virus, unless cells were subsequently treated with UV (Fig. 1B, bottom). These results are in accord with earlier studies showing that, in some cells, the Fhit signal pathway must be induced by treatments causing DNA replication stress and DNA damage (28).

**Fhit is important for checkpoint response after UV exposure.** UV exposure causes a DNA damage response checkpoint that involves recruitment of Rad9-Rad1-Hus1 to damaged chromatin and activation of Atr and Chk1 (29). Under experimental conditions as described for Fig. 1, analysis of checkpoint protein expression showed that introduction of FHIT-Wt, but not the FHIT-Y114 mutants, into PFB cultures resulted in increases of Hus1 and pChk1(Ser317) protein expression, with or without exposure to UV (Fig. 2A). The phosphorylation level of histone H2AX was increased after exposure to UV following infection with each of the viruses, likely due to UV damage rather than Fhit expression (Fig. 2A). Similar immunoblot analyses of protein expression in TE14 and TE4.1 cells showed quite different results (Fig. 2B and C).

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**Figure 1.** Cell cycle profile after adFHIT infections. PFBs and Fhit-deficient TE14 esophageal cancer cells were analyzed at 72 hours postinfection at MOI 30. A, infected cells were lysed and 10 μg of protein subjected to SDS-PAGE, transferred to membrane, and probed with antisera, as indicated. Fhit mutant proteins show mobility variation due to charge differences. B, infected cells, with or without UV exposure, were analyzed by flow cytometry.
After infection with the same four viruses, or UV treatment followed by virus infection, expression of Hus1 protein was apparent in FHIT-Wt-infected TE14 and TE4.1 cells after exposure to UV. Expression of Hus1 in the other esophageal cancer cells after infection with the four viruses was variable (not shown) but each UV-exposed FHIT-Wt-infected cell expressed abundant Hus1 (Fig. S2A). Immunoblot analysis of TE14 and TE4.1 cells showed that exposure to UV resulted in expression of pChk1(Ser317) in cells infected with GFP or mutant FHIT viruses, but Chk1 was low and pChk1 absent in UV-treated cells infected with FHIT-Wt virus (Figs. 2B and C), suggesting that Fhit overexpression resulted in reduced expression of Chk1 and pChk1. The data were consistent with results of examination of the other esophageal cancer cells (Fig. S2A and B), indicating that wild-type Fhit protein plays a role in modulation of UV-induced expression of pChk1 level. Increasing the adFHIT multiplicity of infection (MOI) in cancer cell infections induced Hus1 protein expression in a dose-dependent manner, which was more apparent in UV-exposed cells, and in parallel resulted in reduction of pChk1 level (Fig. S2B). In PFB, the introduction of FHIT induced Hus1 expression and Chk1 phosphorylation (Fig. S2B). To determine if Chk1 and pChk1 levels are altered at earlier time points after introduction of Fhit, the levels of pChk1 and Chk1 were assessed in a time-course experiment (Fig. S3A and B). In PFB, pChk1 increased to 2.5-fold at 24 hours and declined to ~2.1-fold from 48 to 72 hours, whereas in cancer cells pChk1 reached a small peak of expression (1.4- to 1.5-fold) at 24 hours, which rapidly decreased in a time-dependent manner (Fig. S3A). At 24 hours, the pChk1 level increased steeply in PFB cells and remained high through 72 hours; pChk1 increased very slightly at 24 hours in cancer cells and was reduced below starting levels by 48 hours (Fig. S3A and B). The data are consistent with the observations at 72 hours (Fig. 2A-C), showing that wild-type Fhit is involved in the modulation of Hus1 expression, followed by induction of pChk1 in PFB; the results suggest that, compared with PFB, the cancer cells are defective in Chk1 activation by phosphorylation but sensitive to Chk1 down-regulation after DNA damage, an important difference between cancer and noncancer cells.

Phosphorylation of Chk1 controls Cdc25A stability through phosphorylation, leading to interaction of Cdc25A with SCF[ubiquitin ligase and acceleration of Cdc25A turnover (30). The resulting reduction of Cdc25A levels is instrumental in slowing progression through S phase, allowing time for DNA repair; if cell elimination is required, apoptosis can be induced through checkpoint termination (30). Recently, it was reported that the balance between cell cycle arrest and induction of apoptosis during the response to genotoxic stress may be regulated through cleavage of Caspase by caspase-7 (31). Caspase is required for phosphorylation and activation of Chk1 protein kinase by Atr-AttrIP complex during DNA replication (32, 33). Thus, we examined Cdc25A and caspase-7 levels in FHIT-infected cells. Phosphorylation of Chk1 increased by 24 hours after adFHIT infection of PFB cells, and down-modulation of Cdc25A level was detected at 24 and 48 hours; caspase-7 expression was consistent with this pattern of Chk1 pathway activation (Fig. 2D, left). In adFHIT-infected TE14 cells, in contrast, Chk1 phosphorylation was minimal, Cdc25A was very slightly reduced, and full-length caspase-7 level was reduced in a time-dependent manner (Fig. 2D, right). The results suggest that phosphorylation of Chk1 plays a role in arrest of cell cycle in PFBs, whereas the pChk1-Cdc25A pathway was abrogated by FHIT infection of cancer cells. Consistent with this interpretation, flow cytometric analysis showed that introduction of adFHIT in esophageal cancer cells, but not in PFBs, resulted in an increase of the sub-G1 population at 48 to 72 hours, whereas UV exposure followed by adFHIT resulted in enhancement of sub-G1 induction in cancer cells and PFBs (Fig. S3C and D). These observations reinforce the proposal that Fhit plays a role in the activation of the checkpoint; in cancerous cells, defects in the Chk1 response after DNA damage would lead to uncoupling of G2/mitosis from completion of S phase, eliciting elimination of damaged cells through apoptosis or mitotic catastrophe (29, 34, 35); in noncancerous cells, checkpoint activation would contribute to repair of damage.

**Fhit modulation of Hus1 expression.** To clarify mechanisms for Fhit modulation of checkpoint pathways, endogenous Fhit knockdown by siRNA was the next step. Fhit knockdown in UV-treated 293 cells resulted in the reduction of Hus1 protein level; Rad1 protein was also apparently reduced (Fig. 3A). Similarly, knockdown of endogenous Rad1 by siRNA introduction led to the reduction of Hus1 protein level; simultaneous knockdown of Fhit and Rad1 by siRNA introduction also resulted in Hus1 reduction (Fig. 3A), whereas levels of Rad9 and Grb2 protein were not altered, confirming the specificity of the siRNA inhibition.
Asin(A proteasome-dependent degradation results in increased Hus1 expression. Pathway and Atr/Chk1 checkpoint responses. (Fig. 3C) Knockdown of endogenous Hus1 resulted in Chk1 phosphorylation expression of Hus1 may alter Atr/Chk1 checkpoint responses. (28, 37), we assessed whether down-modulation of DNA-dependent protein kinases (39–41). After exposure of PFBs to role in recognition of DNA damage as substrate of Atr, Atm, and protein composed of 70-, 32-, and 14-kDa subunits, which plays a role in replication, repair, and checkpoint response to Chk1 phosphorylation. Endogenous Hus1 was knocked down in 293 cells by siRNA introduction and proteins were immunoblotted.

On treatment with MG-132, an inhibitor of proteasome-dependent degradation, Hus1 reduction was inhibited (Fig. 3B). These results are consistent with the report that Hus1 level is regulated through proteasome-dependent degradation and that Rad1 inhibits proteasome-dependent Hus1 degradation (36), and suggest that Fhit contributes to this action of Rad1.

In view of previous reports showing that Fhit protein–deficient cells show stronger S and G2 checkpoint responses than wild-type counterparts, presumably through modulation of the Atr/Chk1 pathway (28, 37), we assessed whether down-modulation of expression of Hus1 may alter Atr/Chk1 checkpoint responses. Knockdown of endogenous Hus1 resulted in Chk1 phosphorylation (Fig. 3C), suggesting that Hus1 may be a link between the Fhit pathway and Atr/Chk1 checkpoint responses.

Altered checkpoint response in esophageal cancer cells. The preceding experiments indicated that Fhit up-modulated both Hus1 and pChk1 expression in noncancerous PFBs, but up-modulated only Hus1 in esophageal cancer cells, suggesting that the checkpoint pathway is altered in the cancer cells. The Rad9-Rad1-Hus1 complex forms a sliding clamp on the replicating DNA strand and functions in replication, repair, and checkpoint response to Chk1 phosphorylation by Atr kinase (29). Earlier studies indicated that Fhit is involved in maintenance of genome integrity at the mid-S checkpoint, through the Atr/Chk1 pathway, and that Fhit deficiency altered the response to DNA damaging agents (28, 37, 38). Replication protein A (Rpa) is a ssDNA-binding heterotrimeric protein composed of 70-, 32-, and 14-kDa subunits, which plays a role in recognition of DNA damage as substrate of Atr, Atm, and DNA-dependent protein kinases (39–41). After exposure of PFBs to UV, immunoblot analysis with anti-Rpa32 detected low-mobility proteins corresponding to hyperphosphorylated Rpa (40, 42). Immunoprecipitation with Hus1 antibody, followed by immunoblot analysis, showed association of Hus1 and phospho-Rpa32 after UV exposure (Fig. 4A), suggesting a role for a Hus1-Rpa complex in response to UV damage. Because up to seven Ser/Thr sites in the NH2 terminus of Rpa32 are phosphorylated in response to DNA damage or cell cycle progression (43, 44), we assessed the involvement of Atr kinase activity in Rpa32 phosphorylation using transfectants of wild-type (Wt) and kinase-dead (kd) Atr mutants (Fig. 4B). In Atr-wt transfectants, phosphorylation of Rpa32 was induced efficiently after UV exposure, but was reduced or inhibited in Atr-kd transfectants (Fig. 4C), suggesting that Atr is involved in Rpa32 phosphorylation after UV exposure.

Immunoprecipitation with anti-Hus1, followed by immunoblot with anti-Rpa32 and Rpa70, detected association of Hus1 with the Rpa complex, composed of Rpa70 and hyper- and hypo-phosphorylated Rpa32, in UV-exposed PFB cells (Fig. 4D, right); this association was not apparent in PFB cells without UV exposure (Fig. 4D, left). In contrast, association of Hus1 with hypophosphorylated Rpa32, but not with Rpa70 or hyperphosphorylated Rpa32, was apparent after UV exposure of TE14 and TE4.1 cells (Fig. 4D, right); without UV exposure, the association was not detected (Fig. 4D, left). The association of Atr with AtriP was substantial before UV exposure, and more apparent after UV exposure and FHIT introduction in PFB cells, whereas this association was reduced or undetectable in TE14 and TE4.1 cells (Fig. 4D), suggesting that the mid-S checkpoint is altered in the cancer cells and that Fhit is involved in modulation of this checkpoint response. Thus, the Atr
staining with Fhit, pChk1 (Ser 317), Hus1, and Bax antisera. Four days later, tissues were fixed and sectioned for immunohistochemical analysis. In mice in which FHIT therapy was applied at 21 days after N-nitrosomethylbenzylamine, when tumors of ~1 mm were apparent, mice were sacrificed and tissues processed and stained 4 days after FHIT therapy. Hus1 expression was induced in the upper layer of epithelium, apparently coincident with the expression of the human FHIT transgene, whereas Hus1 expression in GFP-infected tissues showed homogeneously weak staining (Fig. 5). pChk1 in GFP-treated mice was detected in highly proliferative, thick portions of epithelium with proliferating cell nuclear antigen (PCNA)–positive regions (PCNA data not shown). In contrast, the FHIT treatment resulted in apparent reduction of pChk1 to an undetectable level in proliferative, thick portions of epithelium in forestomach tissue; pChk1 was detectable in low proliferative portions of marginal epithelium, suggesting that the genotoxin-induced damage disturbed the S-phase checkpoint response and that introduction of FHIT led to down-regulation of Chk1 in damaged or cancerous cells, triggering induction of programmed cell death (35). Consistent with this, proapoptotic Bax staining was induced after FHIT virus administration compared with GFP-treated mice, as previously reported (22).

**Proposed roles of Fhit in normal and cancer cells.** In summary, we have defined functions for wild-type Fhit in the DNA damage checkpoint response. The introduction of FHIT modulated the checkpoint response through the modulation of Hus1 expression and Chk1 activation. In esophageal cancer cells with dysfunctional S-phase checkpoint, exogenous Fhit expression resulted in altered responses, poor induction, and rapid down-modulation of Chk1 phosphorylation, triggering cell death in cancer cells (see model in Fig. 6, summarizing the proposed roles of Fhit in normal and cancer cells). Although delineation of the exact role of the Fhit Y114 residue, whether phosphorylated or unphosphorylated, requires further study, it is clear that amino acid substitution at Y114 drastically alters Fhit function in modulation of the DNA damage response and induction of apoptosis. The data strongly suggest that Fhit prevents tumorigenesis by participation in the

To assess the in vivo significance of Fhit-mediated modulation of the mid-S checkpoint pathway, N-nitrosomethylbenzylamine-induced lesions of Fhit−/− mice, which were untreated or treated by FHIT virus oral therapy, were examined. Fhit expression was confirmed by immunohistochemical analysis. In mice in which FHIT therapy was applied at 21 days after N-nitrosomethylbenzylamine, when tumors of ~1 mm were apparent, mice were sacrificed and tissues processed and stained 4 days after FHIT therapy. Hus1 expression was induced in the upper layer of epithelium, apparently coincident with the expression of the human FHIT transgene, whereas Hus1 expression in GFP-infected tissues showed homogeneously weak staining (Fig. 5). pChk1 in GFP-treated mice was detected in highly proliferative, thick portions of epithelium with proliferating cell nuclear antigen (PCNA)–positive regions (PCNA data not shown). In contrast, the FHIT treatment resulted in apparent reduction of pChk1 to an undetectable level in proliferative, thick portions of epithelium in forestomach tissue; pChk1 was detectable in low proliferative portions of marginal epithelium, suggesting that the genotoxin-induced damage disturbed the S-phase checkpoint response and that introduction of FHIT led to down-regulation of Chk1 in damaged or cancerous cells, triggering induction of programmed cell death (35). Consistent with this, proapoptotic Bax staining was induced after FHIT virus administration compared with GFP-treated mice, as previously reported (22).

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A high level of phosphorylation of Chk1, a survival kinase downstream of Atr, detected in normal but not cancer cells, suggests that Fhit plays distinct roles in response to DNA damage at mid-S in normal and esophageal cancer cells; the reduction of Chk1 phosphorylation level plays a role in induction of apoptosis after FHIT introduction. Discovery of the differential response to exogenous Fhit expression in cancer and noncancer cells led us to examine relevance to Rpa phosphorylation; insufficiency of Rpa phosphorylation in esophageal cancer cells, parallel to Atr-AtrIP association status, illustrated that the Atr-controlled checkpoint response and downstream Chk1 and Rpa modulation are different in normal and esophageal cancer cells. The cumulative results thus far suggested that the dysfunctional Atr pathway in cancer cells could be insufficient to activate Chk1 to slow the cell cycle. Moreover, the marked reduction of Chk1 in esophageal cancer cells after UV exposure and FHIT introduction suggested that the Chk1 down-regulation process is different in cancer and noncancer cells, contributing to termination of checkpoint arrest and sensitization to apoptosis induction in cancer cells.

**Fhit activation of checkpoint and apoptosis in vivo.** The mouse forestomach is analogous to the human lower esophagus.
activation of the Atr-controlled checkpoint response. Alterations of the \textit{FRA3B/FHIT} locus due to DNA damage or replication stress, caused by intrinsic or extrinsic agents, may be the first step in early neoplasia, followed by mutations that allow further escape of cells from checkpoint control, including defects in the Atr/Atm-Chk1/2-p53 pathway, which allow cell proliferation, survival, increased genomic instability, and tumor progression. Thus, Fhit may be the first line of defense against the earliest stages in cancer development, suggesting that Fhit replacement or therapeutic activation of the Fhit pathway could contribute to cancer prevention.

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