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Characterization of the role of Fhit in suppression of DNA damage

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Abstract

The fragile histidine triad protein, Fhit, has a number of reported tumor suppressive functions which include signaling of apoptosis in cancer cells *in vitro* and *in vivo*, modulation of the DNA damage response, down-regulation of target oncogene expression, suppression of tumor growth *in vivo*, and suppression of cancer cell invasion and metastasis. Most of these functions of Fhit have been observed on exogenous re-expression of Fhit in Fhit-negative cancer cells. However, little is known about the tumorigenic changes that occur in normal or precancerous cells following loss of Fhit expression. Recently, we have shown that shortly after loss of Fhit expression, cells exhibit signs of DNA replication stress-induced DNA damage and develop genomic instability. Here, we extend these findings through investigation of different factors that affect Fhit function to prevent DNA damage. We found that Fhit activity is dependent upon a functional HIT domain and the tyrosine-114 residue, previously shown to be required for tumor suppression by Fhit. Furthermore, Fhit function was shown to be independent of exogenous and endogenous sources of oxidative stress. Finally, Fhit function was shown to be dependent upon Chk1 kinase activity, but independent of Atr or Atm kinases. Evidence suggests that Fhit and Chk1 kinase cooperate to prevent replication stress-induced DNA damage. These findings provide important and unexpected insights into the mechanism whereby loss of Fhit expression contributes to cell transformation.

Introduction

Genomic instability is an important characteristic of cancer as it enables cells, through increases in mutation frequency, to acquire the numerous phenotypes that contribute to development of the malignant state (Hanahan and Weinberg, 2011). There are various types of genomic instability, but the most commonly observed form in cancer cells is

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chromosomal instability. In precancerous cells, chromosomal aberrations are first detected at common fragile sites, with fragile sites FRA3B and FRA16D being the most frequently affected loci (Tsantoulis et al., 2008). Because these fragile sites are exquisitely sensitive to replicative stress, it is thought that genomic instability originates in precancerous cells because of an increase in DNA replication stress (Gorgoulis et al., 2005; Di Micco et al., 2006). In support of this hypothesis, exposure to chemical agents that cause replication stress generates the same pattern of micro-deletions within FRA3B that is observed in transformed and tumor cells *in vitro* (Durkin et al., 2008). In later stages of cancer development, genomic instability spreads throughout the genome primarily due to a combination of factors including telomere shortening, oxidative stress and defective DNA damage response (Negrini et al., 2010).

Notably, FRA3B and FRA16D reside within the FHIT and WWOX genes, respectively, and deletions within FHIT and WWOX are common in tumor cells and can lead to reduction or loss of Fhit and Wwox protein expression (Durkin and Glover, 2007; Guler et al., 2005). It has been estimated that FHIT is the most frequently altered gene in cancer, rivaling the TP53 gene. In addition to the frequency and clonal pattern of genetic alterations at the FHIT locus in cancer cells, there is considerable phenotypic evidence that Fhit is a *bona fide* tumor suppressor. First, Fhit knockout mice develop more spontaneous tumors and dramatically more carcinogen-induced tumors than wild-type littermates (Zanesi et al., 2001), and viral-mediated FHIT gene delivery prevents and can even cause regression of carcinogen-induced tumors (Ji et al., 1999; Ishii et al., 2001; Dumon et al., 2001). Secondly, over-expression of Fhit in Fhit-negative cancer cell lines suppresses tumorigenicity of xenografts in mice (Siprashvili et al., 1997; Roz et al., 2002; Sevignani et al., 2003). Mechanistically, Fhit suppresses tumor formation by activating apoptotic pathways and down-regulating expression of target oncogenes. In response to oxidative stress, Fhit interacts with ferrodoxin reductase in mitochondria to enhance reactive oxygen species production followed by caspase-3 activation and apoptosis (Trapasso et al., 2008; Pichiorri et al., 2009; Okumura et al., 2009). In response to genotoxic stress, Fhit modulates the DNA damage response such that cancer cells commit to apoptosis (Saldivar et al., 2010). There is also evidence that Fhit can affect calcium release from mitochondria and thereby promote apoptosis (Rimessi et al., 2009). Finally, Fhit has been shown to negatively regulate the epithelial-to-mesenchymal transition and to minimize the invasiveness and metastatic potential of cancer cells (Jayachandran et al., 2007; Joannes et al., 2010).

Because of reports that Fhit-negative cells have altered DNA damage responses, increased survival following genotoxic insult and a higher frequency of mutations, Fhit has been called the guardian of the preneoplastic genome, acting to eliminate genetically-altered cells through apoptosis and thereby guard against tumorigenic changes (Pichiorri et al., 2008). However, Fhit has recently been shown to have a more direct role in maintaining genomic stability (Saldivar et al., 2012). In this study, it was shown that upon loss of Fhit expression, cells are unable to efficiently replicate DNA due to a failure to adequately synthesize thymidine triphosphate pools. As a result, Fhit-deficient cells accumulate stalled replication forks and spontaneous DNA double-strand breaks (DSBs) culminating in chromosome aberrations and acquisition of cancerous phenotypes. Examples of the chromosome alterations developed following Fhit loss include chromosome breaks and gaps, sister-chromatid exchanges, and changes in chromosome number (for examples, Fig. 1). From these findings, an attractive model for the initiation of genomic instability in preneoplastic cells through loss of Fhit expression was proposed (Saldivar et al., 2012). We now further characterize the cellular processes through which Fhit suppresses the formation of spontaneous DNA damage.

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Materials and methods

Cell lines and reagents

HEK293, H1299 and kidney epithelial cells established from wild-type and Fhit knockout C57Bl/B6 mice were cultured in MEM medium supplemented with 10% FBS and 100 μg/ml gentamicin. Cells were treated for 4 h with the Chk1 inhibitor, SB218078, at a final concentration of 2 μM. Cells were treated for 4 h with the ATR inhibitor, ATR-45, at a final concentration of 10 μM. Cells were treated with N-acetyl-L-cysteine (NAC) at a final concentration of 3 mM.

Plasmids and siRNA transfections

siRNAs were transfected into HEK293 cells as previously described (Saldivar et al., 2012). In brief, 1 μg of FHIT siRNA or control siRNA was mixed with 5 μl of Lipofectamine (Invitrogen) and diluted in Opti-MEM (Gibco) and added to cells. Two days following transfections, Fhit expression was assessed to confirm knockdown. RcCMV-Flag plasmids with FHIT wild-type cDNA, FHIT-H96N mutant cDNA or FHIT-Y114A mutant cDNA were transfected in H1299 cells using Lipofectamine 2000 (Invitrogen) according to the protocol provided by the manufacturer.

Comet assays

Neutral comet assays were performed using the CometAssay kit (Trevigen) as previously described (Saldivar et al., 2012).

Results and discussion

The Fhit-substrate complex

Fhit is a member of the HIT family of nucleoside hydrolases and transferases. Specifically, Fhit catalyzes hydrolysis of two dinucleosides, diadenosine triphosphate and diadenosine tetraphosphate, *in vitro* (Barnes et al., 1996), though the *in vivo* substrate relevant to Fhit biological functions is not known. Fhit mutants that are unable to bind candidate dinucleoside substrates in *in vitro* biochemical assays, are unable to participate in known *in vivo* Fhit functions, such as apoptosis, binding to chaperones for transport to mitochondria and interaction with mitochondrial feredoxin reductase to modulate production of reactive oxygen species (Pichiorri et al., 2009). Furthermore, these Fhit mutants fail to suppress tumor growth in mice or cause apoptosis in tumor cells *in vitro* (Siprashvili et al., 1997; Trapasso et al., 2003). To determine if prevention of DSBs by Fhit is also dependent upon enzymatic or substrate-binding activity of Fhit, we transiently transfected Fhit-negative H1299 cells with pRcCMV plasmids for expression of wild type (wt) Fhit, a His96Asn Fhit mutant (H96N) with increased substrate binding and decreased catalytic activity, or a Tyr114Ala mutant (Y114A) with very poor substrate-binding affinity (Trapasso et al., 2003; Pichiorri et al., 2009). Immunoblotting and indirect immunofluorescence confirmed expression and equivalent transfection efficiencies for all plasmid transfections (Fig. 2A–C). To measure DSBs we performed neutral comet assays on the transfected H1299 cells and measured the comet tail moment of individual cells, an indirect measurement of the degree of DNA DSBs. The results revealed that the Y114A mutant was completely unable to suppress spontaneous DNA breaks compared to the empty vector control (Fig. 2D), while the H96N mutant reduced DSBs to a level intermediate between wt Fhit and empty vector control. Similar results were obtained in Fhit-negative MKN74 stomach cancer cells with stable expression of Fhit wt, H96N mutant, or the empty vector control. Thus, as is the case with Fhit and Fdxr interaction, induction of apoptosis and tumor suppression, a Fhit-substrate complex participates in preventing spontaneous DNA damage.

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Interestingly, other members of the HIT family of nucleotide hydrolases and transferases, including Aprataxin and Hint1, have reported roles in DNA repair (Martin et al., 2011). Aprataxin is encoded by the APTX gene, which is mutated in patients with the neurological disorder, ataxia-ocular apraxia (Date et al., 2001; Moreira et al., 2001). Aprataxin associates with DNA repair proteins XRCC1 and PARP-1 (Sano et al., 2004; Gueven et al., 2004) and removes abortive DNA ligation intermediates, allowing ligation and repair of ssDNA nicks and gaps (Ahel et al., 2006). Hint1 reportedly participates in repair of DSBs, through interaction with and modulation of γH2AX and ATM signaling in response to ionizing radiation-induced DSBs (Li et al., 2008). HIT proteins have apparently evolved functions necessary for maintaining genomic integrity, and thus, the Fhit-substrate complex may participate in an important reaction to promote DNA replication progression. In accord with this hypothesis, a mutant of Hnt2, the Fhit homolog in *Saccharomyces cerevisiae*, interacts negatively with mutants of multiple genes involved in response to DNA damage and replication, including Pol3, the catalytic subunit of DNA polymerase δ (Constanzo et al., 2010).

Exogenous and endogenous oxidative stress

Oxidative stress can cause DNA damage, most notably the oxidation of guanine to form 8-oxo-2'deoxyguonosine (Cooke et al., 2003; Evans et al., 2004), and reducing the reactive oxygen species (ROS) burden on a cell prevents accumulation of DSBs (Sung et al., 2010). Fhit is known to regulate ROS production in response to oxidative stress through interaction with Fdxr in mitochondria. We investigated whether Fhit-deficient cells develop DSBs because of increased DNA oxidation. First, we cultured kidney epithelial cells established from $Fhit^{+/+}$ or $Fhit^{-/-}$mice in the presence or absence of N-acetyl-L-cysteine (NAC), an antioxidant, for 40 h and measured DNA breaks with the neutral comet assay. While NAC treatment reduced DNA damage in $Fhit^{+/+}$ cells, it had no effect on $Fhit^{-/-}$ cells (Fig. 3A), suggesting that Fhit does not protect against DNA damage caused by endogenous ROS. Next, we cultured $Fhit^{+/+}$ or $Fhit^{-/-}$epithelial cells in normal atmospheric conditions (20% O_2) or in a low oxygen environment (5% O_2) closer to physiological oxygen levels. After 48 h, the degree of DSBs was evaluated using the neutral comet assay. We observed no difference in the spontaneous DSBs when cells were grown in the low oxygen environment compared to the normal growth conditions (Fig. 3B). We conclude that the spontaneous DSBs in Fhit-deficient cells are not caused by an increase in oxidative stress.

Fhit and the DNA damage checkpoint kinases

In response to DNA damage, cells activate an elaborate response to arrest cell cycle progression, signal repair and choose whether to restart cell proliferation, remain in a state of senescence, or commit to apoptosis (Branzei and Foiani, 2009). This response is collectively referred to as the DNA damage response (DDR). Central to the DDR are the checkpoint kinases ATM, ATR and Chk1. Chk1 is a checkpoint kinase that responds to fork stalling during S/G2 phase during replication. It regulates cell-cycle checkpoint to arrest cells in S and G2 phases (Dai and Grant, 2010). ATR plays a role at stalled forks and double strand breaks by activating Chk1 via phosphorylating Chk1 (Guo et al., 2000; Liu et al., 2000; Cimprich and Cortez, 2008). ATM also acts by phosphorylating multiple targets in response to DSBs (Lavin, 2008). While these 3 kinases have overlapping roles, they also perform separate specific functions.

Knowing that Fhit prevents spontaneous DSBs, specific small molecule inhibitors were used to inhibit each of these kinases individually in HEK293 cells, and comet assays were used to measure DNA breaks. The results shown in Fig. 4A show that Chk1 inhibition led to an increase in DSBs in control HEK293 cells. This increase was similar to the level of DNA breaks in untreated, Fhit-silenced HEK293 cells. In contrast, Chk1 inhibition in Fhit-

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deficient HEK293 cells had little effect on DNA damage, suggesting that Fhit function to prevent DNA damage is dependent on Chk1 activity. ATR and ATM inhibitors were also used, however there was no dependency of Fhit function on either of these kinases (Fig. 4B and data not shown). Similarly, Chk1 inhibition led to an increase in DNA breaks in Fhit$^{+/+}$ mouse kidney cells, but caused no observable increase in damage in Fhit$^{-/-}$mouse kidney cells (Fig. 4C). Because Fhit only suppresses DSBs when Chk1 activity is not inhibited, and Chk1 only suppresses DSBs in Fhit-expressing cells, we conclude that Fhit and Chk1 activities are co-dependent.

The observed co-dependency for Fhit and Chk1 is intriguing as Fhit and Chk1 appear to have some overlapping roles during S phase. For example, both Fhit and Chk1 promote fork progression during unperturbed S phase (Petermann et al., 2006; Saldivar et al., 2012); both prevent DNA damage during S phase (Gagou et al., 2010); and both prevent chromosome instability. Furthermore, the tumor spectrum in Chk1$^{+/-}$Chk2$^{-/-}$mice partially phenocopies that of Fhit$^{-/-}$mice (Niida et al., 2010, Zanesi et al., 2001). It is not entirely clear how Chk1 promotes replication fork movement, but it is likely that Chk1 phosphorylates one or more proteins necessary for DNA replication. Of interest, Fhit has 2 motifs that are predicted to be phosphorylation targets of Chk1. Thus, a possible scenario to explain the co-dependency would be that Chk1 phosphorylates Fhit, leading to its activation to promote DNA replication. Inhibition of Chk1 kinase activity would lead to diminished Fhit function; in the absence of Fhit expression, Chk1 lacks its target to promote fork progression.

Conclusion

There are a number of reports concerning suppression of tumorigenicity of xenografts after FHIT gene expression, numerous reports of exogenous expression causing apoptosis of recipient cells, and of FHIT gene therapy of xenografts; most of these studies showed that Fhit caused suppression or regression of tumors in preclinical models through apoptosis. We have shown that loss of expression of Fhit protein causes DNA damage and genome instability and expression of Fhit reduces this DNA damage and genome instability. In this report, we have further shown that these functions of Fhit are dependent upon the Fhit-substrate complex, as are other tumor suppressive functions of Fhit. However, it is clear that tumor suppression cannot occur through reduction of DNA damage in a recipient tumor cell because the DNA alterations have already occurred. Thus, it is likely that Fhit contributes to suppression of tumor initiation and progression through its role in protection of genome stability and that it has a second role in suppressing tumorigenicity through apoptosis, perhaps of cells that have sustained DNA damage. The finding that Fhit and Chk1 have co-dependent activities to minimize DNA damage suggests that loss of Fhit expression in the earliest preneoplastic lesions impairs one of the branches of the S phase checkpoint setting the stage for future tumorigenic events.

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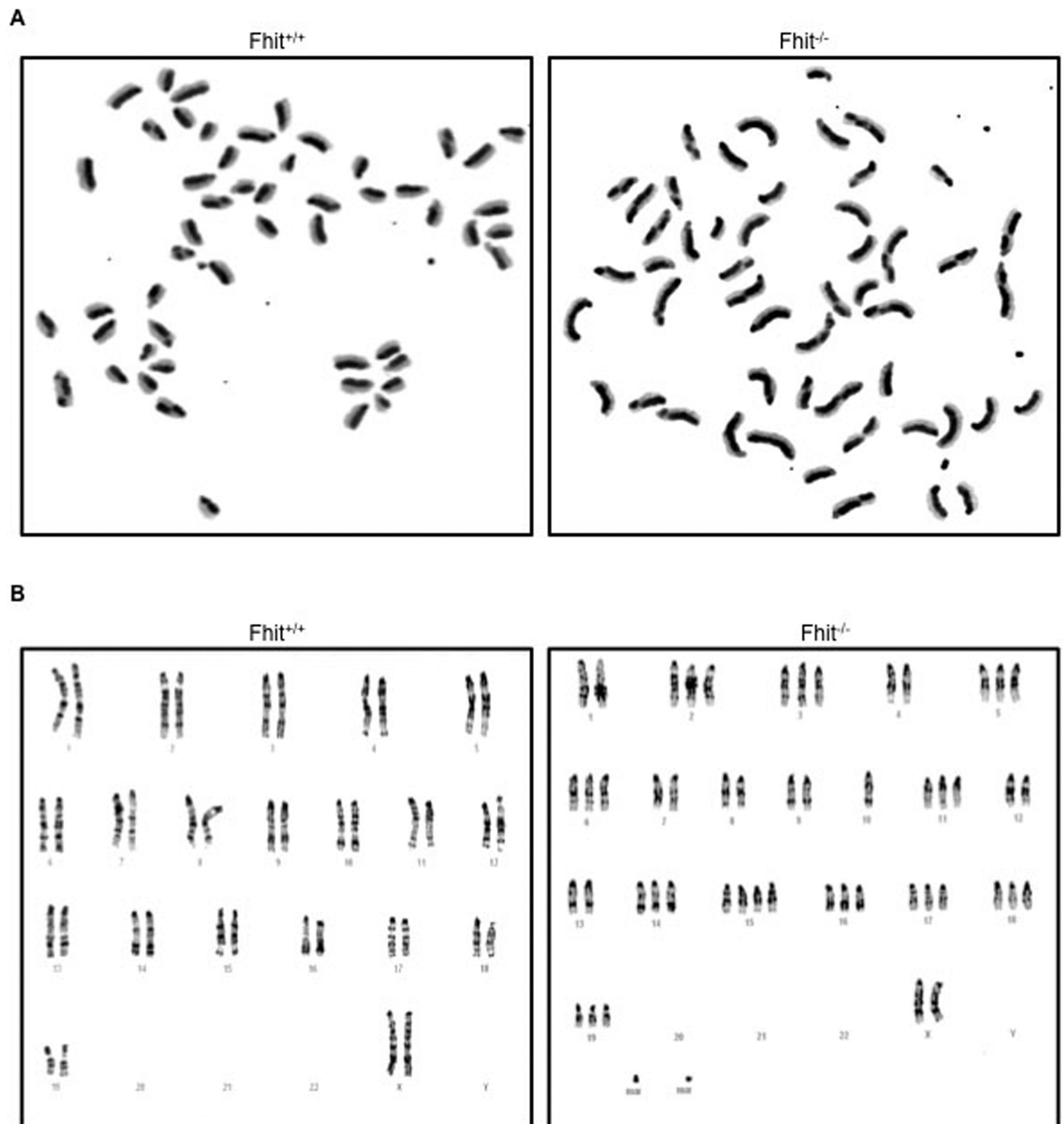

Fig. 1.

Examples of chromosome aberrations in Fhit-deficient cells. A. Representative images of sister chromatid exchanges (SCEs) in $Fhit^{+/+}$ and $Fhit^{-/-}$mouse epithelial cells. On average, $Fhit^{-/-}$cells have more than twice as many SCEs/metaphase than $Fhit^{+/+}$ cells. B. Representative karyotype image of $Fhit^{+/+}$ and $Fhit^{-/-}$ mouse epithelial cells illustrating an abnormal chromosome number in Fhit-negative cells.

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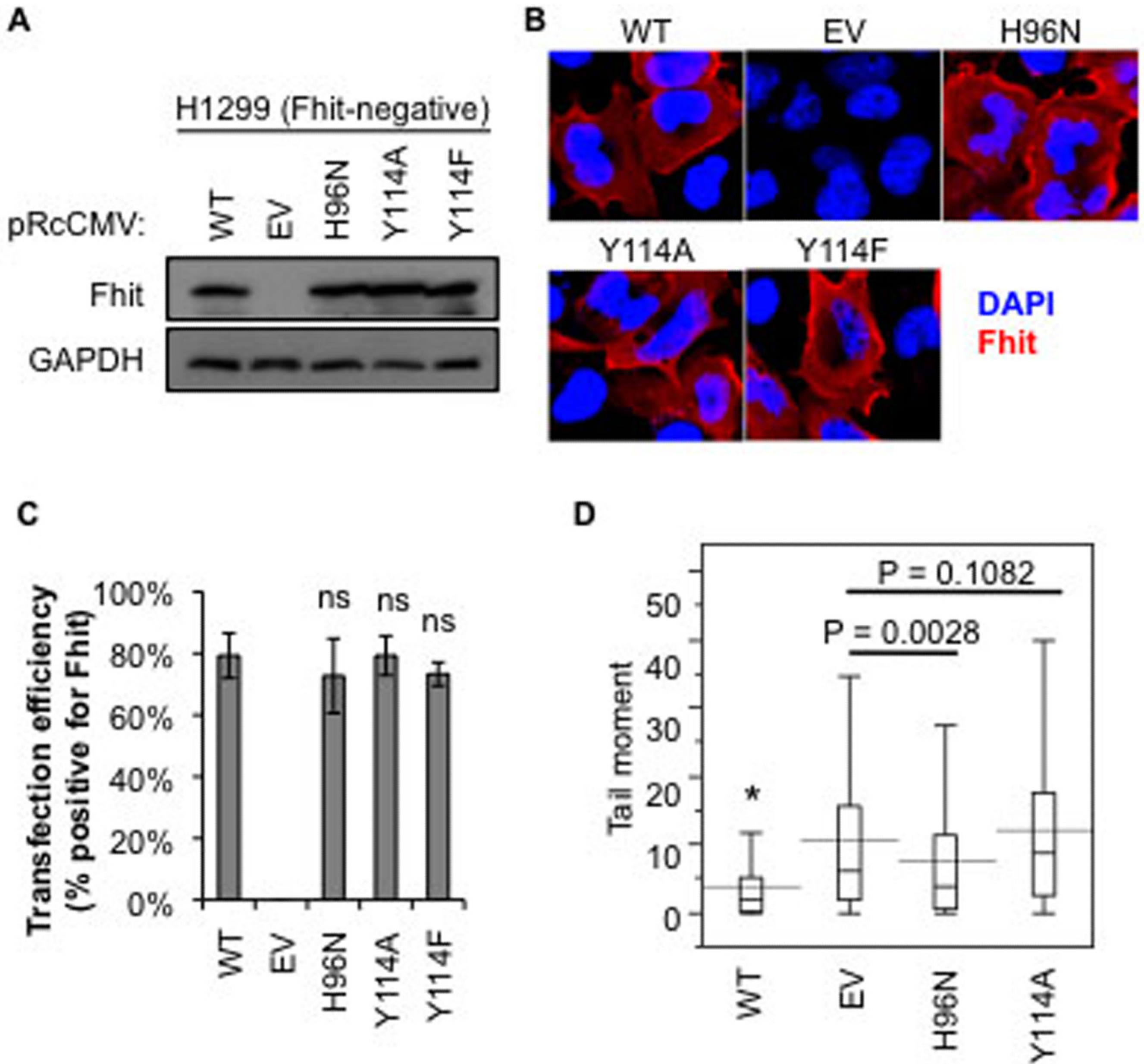

Fig. 2.

The Fhit-substrate complex suppresses DNA breaks. A. Western blots of Fhit and GAPDH expression in H1299 cancer cells 3 days after transient transfection with pRcCMV-FHIT-wt (wt), pRcCMV-empty vector (ev), pRcCMV-FHIT-H96N mutant (H96N), pRcCMV-FHIT-Y114A mutant (Y114A), or pRcCMV-FHIT-Y114F mutant (Y114F). B. Immunofluorescence of Fhit expression in H1299 cells transfected as in (A); bars, 20 μm. C. Transfection efficiency of H1299 cells transfected as in (A). Transfection efficiencies were determined as (Fhit-expressing cells/total cells)×(100%) using immunofluorescence images obtained as in (B). D. Neutral comet assays and box plots of tail moments of H1299 cells transfected as in (A). Statistical significance was determined using the Kruskal-Wallis rank sum test; *$P < 0.005$ for each comparison.

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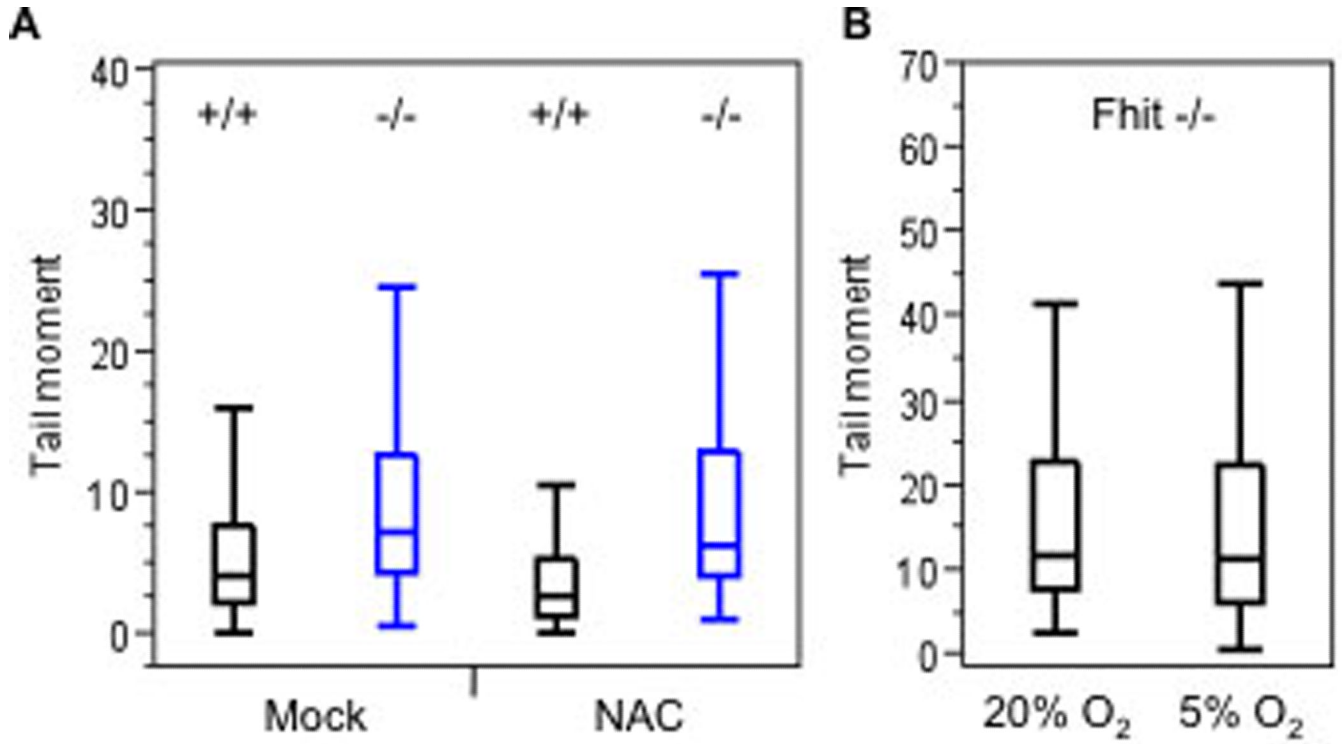

Fig. 3.
DNA damage in Fhit-deficient cells is not caused by oxidative stress. A. Neutral comet assays and box plots of tail moments of $Fhit^{+/+}$ and $Fhit^{-/-}$mouse kidney epithelial cells treated with N-acetyl-L-cysteine (NAC), 3 mM for 40 h, or mock treated. B. Neutral comet assays and box plots of tail moments of $Fhit^{+/+}$ and $Fhit^{-/-}$mouse kidney cells grown in an ambient atmospheric environment (20% oxygen) or in a low oxygen environment (5% oxygen).

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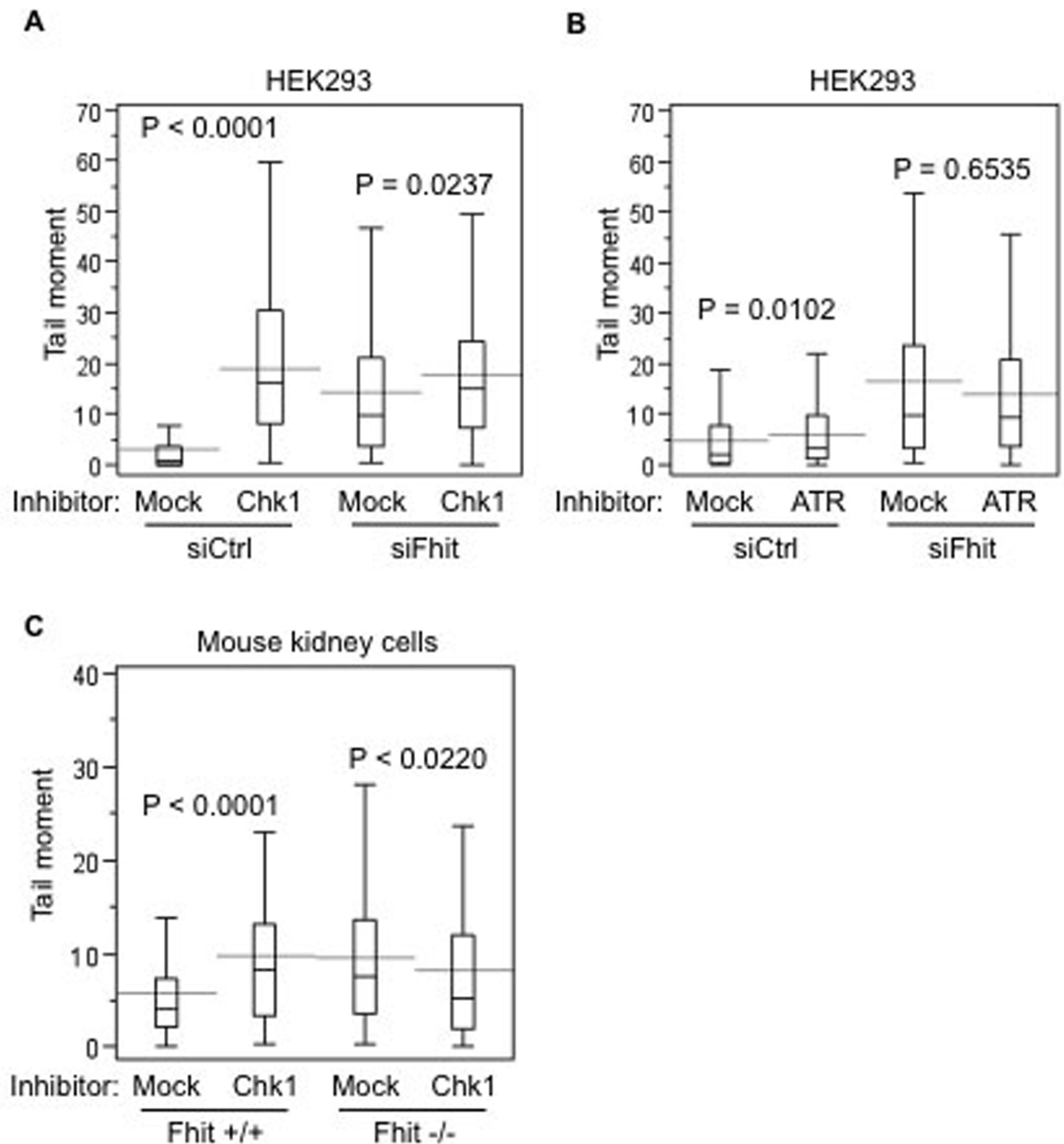

Fig. 4.

Fhit function is dependent upon Chk1 activity. A and B. Neutral comet assays and box plots of tail moments of control or FHIT-silenced HEK293 cells treated for 4 h with a Chk1 inhibitor (A) or an ATR inhibor (B). C. Neutral comet assays and box plots of tail moments of $Fhit^{+/+}$ and $Fhit^{-/-}$ mouse kidney cells treated for 4 h with a Chk1 inhibitor. P-values were calculated using the Kruskal-Wallis rank sum test.