The Origin of Genome Instability in Cancer: Role of the Fragile Site Gene Product FHIT

Dissertation

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By

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The transformation of normal cells to cancer cells involves multiple steps mediated by the acquisition of mutations, selection and clonal expansion of cells with favorable mutations. Most cancers exhibit remarkable genomic instability, defined as an elevated rate of genetic mutation at the single nucleotide and chromosome levels. Genomic instability is a facilitating hallmark of cancer in that it raises the probability of generating cancer-promoting mutations. Multiple factors contribute to the genome instability phenotypes seen in cancer, but the molecular processes initiating instability in sporadic cancer are unknown. In dysplastic cells, genomic alterations are first seen at chromosome fragile sites. These fragile sites are exquisitely sensitive to agents that stress DNA replication forks, and thus, it is thought that replicative stress is a major source of genome instability in cancer.

A frequent and very early genetic alteration in precancerous cells is deletion within fragile site FRA3B, which overlaps exons of the large FHIT gene, resulting in loss of FHIT protein expression. Here it is shown that loss of FHIT expression triggers endogenous replication stress hindering replication fork progression and inducing fork stalling and collapse. Consequently, FHIT-deficient cells develop spontaneous DNA breaks and chromosome instability. Mechanistically, FHIT loss-induced replication stress
is due to an imbalance in the deoxyribonucleotide triphosphate pool and an insufficient supply of thymidine triphosphate. FHIT up-regulates the S-phase-specific expression of thymidine kinase 1, a component of the pyrimidine salvage pathway for the production of thymidine triphosphate. Balanced precursors of DNA are needed for efficient and accurate DNA replication, and notably restoration of nucleotide balance rescues DNA replication defects in FHIT-deficient cells.

Under selective pressure, FHIT-deficient clones enabled by oncogenic mutations emerge with newly acquired precancerous phenotypes, suggesting that FHIT loss-induced genome instability facilitates tumorigenesis. Collectively, these findings support a model where loss of FHIT expression initiates genomic instability in dysplastic lesions, linking alterations at chromosome fragile sites to the origin of genome instability and cancer progression.
Dedicated to Catherine Waters for her love and companionship

To my mother for her love and support, for valuing my education, and for cultivating my curiosity

To my father for teaching me to work hard, to be courageous, to never quit, and inspiring me to always do my best

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Chapter 1. FHIT, a Guardian of the Preneoplastic Genome?

Contains portions of a published manuscript (Saldivar et al., 2010, *Journal of Cellular Biochemistry* 109(5):858-865)

1.1 Introduction

Common chromosome fragile sites are large, unstable regions of the human genome that are highly sensitive to genotoxic stress and perturbation of replication (Durkin and Glover, 2007). They are “common” in the sense that they can be induced in normal cells from all individuals, as opposed to the “rare” fragile sites that are only inducible in individuals with a specific inherited genetic defect such as Fragile X syndrome, which is caused by a CGG-repeat expansion. Common fragile sites were discovered in the early 80’s, and it was noted that many of them mapped to loci that are non-randomly altered in cancers (Yunis and Soreng, 1984). Thus, it was thought that cloning of fragile sites would lead to discovery of genes that contribute to cancer development through genomic alteration. In 1996, the first gene spanning a common fragile site was reported and was named *FHIT* (fragile histidine triad) because the gene locus was at a fragile site and the sequence possessed homology to members of the histidine triad family (HIT) (Ohta et al., 1996). However, after *FHIT* was cloned and characterized as a cancer suppressor, it was suggested that *FHIT* and other fragile genes
are altered in cancers due to their exquisite stress sensitivity rather than to a selective advantage imparted by loss of expression of fragile gene products (Le Beau et al., 1998). More than 17 years and >900 scientific publications after the discovery of the fragile FHit gene, there are still questions to pursue concerning the selective advantage conferred to cells by deletions within the FHit locus. Are these regions frequently deleted in cancers because they are most sensitive to DNA replication stress, or does the deletion drive the expansion of the preneoplastic cell in which the alteration occurred, or do both mechanisms contribute?

1.2 The FHit gene and gene product

The FHit gene is located at 3p14.2 and is a large 10-exon gene of ~1.5 Mb in length. In contrast, FHit messenger RNA is only 1.1 kb with exons 5-9 encoding a small protein of 147 amino acids (Huebner et al., 1998). FHit spans FRA3B (Figure 1.1A), the most active of the common fragile sites in that it exhibits the highest sensitive to replication inhibitors in lymphoblasts (Durkin and Glover, 2007). The core of FRA3B overlaps FHit exons 4-6 and the majority of FRA3B alterations occur within this region (Figure 1.1B) (Durkin et al., 2008). Aberrant transcripts of FHit are detected at a high frequency in cancer cells, many of them due to deletions within the FRA3B core region, and reduced or absent FHit protein expression occurs in nearly 50% of all cancers. Silencing of FHit expression can also occur by CpG methylation within the promoter, and this mode of FHit expression silencing is a frequent occurrence in cancer cells. FHit loss occurs most commonly in epithelial-derived cancers of the lung, esophagus,
breast, throat, stomach, skin, pancreas, cervix and kidney; though there are examples of FHit loss in many other types of cancer (Huebner and Croce, 2001).

**Figure 1.1. The FRA3B/FHIT locus.** (A, left panel) Fluorescent image of chromosome 3 showing the gap at fragile site, FRA3B. Fragile sites are seen as gaps or breaks on mitotic chromosomes following mild replication stress. (A, right panel) Fluorescent in situ hybridization (FISH) detection of the FHIT locus at FRA3B. Yellow arrows point to the visible gap. (B) Schematic of the FHIT gene at human chromosome 3p14.2. Numbers 1-10 designate the 10 exons, and the black shaded exons 5-9 indicate the protein-coding exons. Horizontal red lines indicate deletion break points commonly seen in cancer cells. The green dotted line marks the FRA3B “core” region where most deletions occur.
FHIT protein possesses homology to members of the histidine triad (HIT) family of proteins. Members of the HIT protein family each contain a His-X-His-X-His-XX sequence motif that forms the core of the protein active site involved in nucleotide binding, hydrolysis or transfer. In addition to the FHIT branch, there are four other branches of the HIT protein family, including Hint, Aprataxin, GalT, and DcpS (Martin et al., 2011). Barnes et al [1996] first reported FHIT to be a diadenosine 5’ ,5’’’-P\(^1\),P\(^3\)-triphosphate (Ap\(_3\)A) hydrolase, though FHIT can also hydrolyze Ap\(_4\)A, albeit with less efficiency (Barnes et al., 1996). The importance of FHIT enzymatic activity is implied from the fact that it has been conserved throughout much of eukaryotic evolution, with FHIT/Ap\(_3\)ase homologs present in yeast, worms, insects and mammals. However, the biological relevance of FHIT enzyme function remains elusive. FHIT subcellular localization is restricted to the cytoplasm and cytosolic organelles including the mitochondrion (Figure 1.2). FHIT protein is expressed in most adult human tissues but is most strongly expressed in the epithelial cells lining most organs. During development, murine Fhit expression is most abundant in organs derived from the endoderm, such as the bronchi, trachea, esophagus, stomach and intestine (Falvella et al., 2000). Interestingly, FHIT protein expression is absent in most human cancers derived from these same tissues.
1.3 Tumor suppressor function of FHIT

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 (Fong et al., 2000; Zanesi et al., 2001), and viral-mediated FHIT gene delivery prevents and can even cause regression of carcinogen-induced tumors (Dumon et al., 2001; Ishii et al., 2003; Ji et al., 1999). Secondly, over-expression of FHIT in FHIT-negative cancer cell lines suppresses tumorigenicity of xenografts in mice (Roz et al., 2002; Sevignani et al., 2003; Siprashvili...
et al., 1997). 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 (Okumura et al., 2009; Pichiorri et al., 2009; Trapasso et al., 2008). In response to genotoxic stress, FHIT modulates the DNA damage response such that cancer cells commit to apoptosis (Figure 1.3) (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 negatively regulates the epithelial-to-mesenchymal transition and minimizes the invasiveness and metastatic potential of cancer cells (Jayachandran et al., 2007; Joannes et al., 2010). Mutational analysis of FHIT reveals that tumor suppression is independent of FHIT enzymatic activity but potentially dependent on FHIT-substrate binding (Trapasso et al., 2003). These studies led to the hypothesis that the FHIT-substrate complex is the active signaling molecule for tumor suppression.
Figure 1.3. Pathways of FHIT tumor suppression. In response to oxidative stress, FHIT is chaperoned into the mitochondrion where it interacts with ferrodoxin reductase and stimulates the release of reactive oxygen species triggering cytochrome C release and activating the caspase cascade. In response to genotoxic stress, FHIT modulates the DNA damage checkpoint protein Chk1, through an unknown mechanism to induce cell cycle arrest and apoptosis in cancer cells. Importantly, FHIT-deficient cells are resistant to oxidative and genotoxic agents and develop preneoplastic changes.

1.4 Loss of FHIT expression in early preneoplasias

Most tumor suppressive functions of FHIT have been described in malignant cells (eg. FHIT induces apoptosis in cancer cell lines and suppresses cancer cell invasiveness). However, there are numerous reports of genetic alterations at the FHIT locus and loss of FHIT protein expression in preinvasive lesions, suggesting a tumor suppressive role for FHIT in the early stages of cancer development (Guler et al., 2005; Michael et al., 1997; Sozzi et al., 1998). In squamous cell carcinoma of the lung, FHIT gene alterations and
loss of FHIT protein expression occurs in up to 90% of dysplastic lesions (Sozzi et al., 1998). The early loss of FHIT in lung cancers may be unique to squamous cell carcinomas of the lung, as a study reported that while FHIT protein was loss in the majority of lung adenocarcinomas, FHIT protein was present in over 90% of atypical adenomatous hyperplasia and in 80% of bronchioloalveolar carcinoma, both thought to represent early stages of lung adenocarcinoma development (Kerr et al., 2004). Squamous cell carcinoma is more prominent in patients with a history of smoking, and the FHIT gene is highly sensitive to environment carcinogens, especially carcinogens present in cigarette smoke (Sozzi et al., 1997; Tseng et al., 1999). FHIT gene alterations and loss of FHIT expression is observed in the histologically normal bronchial epithelium of chronic smokers (Mao et al., 1997). FHIT loss may be an initiating event in development of lung tumors of carcinogen-exposed individuals. In sporadic breast cancer, FHIT loss also occurs in the preinvasive stages, primarily seen at the preinvasive dysplastic stage with estimates of FHIT loss occurring in as many as 70% of ductal in situ carcinomas; LOH at the FHIT locus is found in as many as 25% of intraductal hyperplasias (Ahmadian et al., 1997; Guler et al., 2005). FHIT loss is also very common in the preinvasive lesions of the esophagus (Kitamura et al., 2001; Mori et al., 2000), cervix (Birrer et al., 1999; Butler et al., 2002), oral cavity (Yuge et al., 2005) and other organ tissues (Luan et al., 1998; Ozkara and Corakçi, 2005; Velickovic et al., 1999), suggesting that in many different types of cancer, FHIT plays an important role in suppressing the formation of the early preneoplastic and premalignant lesions.
1.5 Genomic instability in FHIT-deficient cells

Preneoplastic lesions frequently exhibit activation of DNA damage responses. DNA damage in precancerous cells is primarily caused by endogenous DNA replication stress due to a dysregulated cell cycle and premature S-phase entry (Gorgoulis et al., 2005). DNA damage in premalignant lesions prompts checkpoint activation, cell cycle arrest, senescence and/or apoptosis. Indeed, many preneoplastic lesions have a low proliferation index and/or high apoptotic index. This checkpoint response to DNA damage forms a barrier to cancer progression and represents an intrinsic mechanism of tumor suppression (Bartkova et al., 2005). It is also one of the major contributors to oncogene-induced senescence, as many oncogenes cause replication stress and DNA damage (Bartkova et al., 2006; Di Micco et al., 2006). The checkpoint barrier to tumorigenesis generates the selective force for clonal expansion of neoplastic cells harboring mutations that impair the DNA damage checkpoints and explains the high-prevalence of TP53 mutations that compromise the pro-apoptotic and pro-senescence functions of p53 in cancer cells (Bartek et al., 2007; Halazonetis et al., 2008).

Alterations at the FHIT locus are coincident with activation of the DNA damage response in preneoplastic cells (Tsantoulis et al., 2008). The simplest explanation for this observation is that replication stress causes DNA damage, specifically at fragile loci including FRA3B/FHIT, triggering activation of the DNA damage response. In this scenario, fragile sites would serve as a means of surveillance for aberrant DNA replication and DNA damage in precancerous cells signaling the checkpoints to arrest further proliferation and prevent further neoplastic changes and cancer progression. In
reality, the situation is more complicated as FHit protein modulates the DNA damage checkpoint response (Ishii et al., 2006) and the S-phase checkpoint following replication stress (Hu et al., 2005). Thus, loss of FHit expression actively influences the DNA damage response and the clonal evolution of precancerous cells with ongoing DNA damage. Indeed, FHit-deficient cancer and normal cells exhibit increased resistance to genotoxic agents, including mitomycin C and UVC, and resistant clones acquire increased mutations, preneoplastic alterations and exhibit hallmarks of transformation (Ishii et al., 2008; Ottey et al., 2004).

Evidence suggests that FHit modulates the S/G2 checkpoint response, as Fhit−/− murine kidney epithelial cells express an overactive ATR/Chk1 response and a stronger S/G2 checkpoint. These result in increased resistance to ionizing radiation and increased mutations in resistant cells (Hu et al., 2005). In another study, it was shown that FHit differentially modulated the DNA damage checkpoint of normal and cancer cells in response to UV exposure (Ishii et al., 2006). In primary fibroblasts, FHit overexpression correlated with activation of Chk1, with or without UV exposure. This presumably would promote efficient DNA replication and allow repair of damaged replication forks and completion of DNA synthesis prior to mitosis, preserving genomic integrity. In contrast, FHit-negative esophageal cancer cells exhibited strong checkpoint activation upon UV exposure allowing cancer cells to cope with the DNA damage. Upon overexpression of FHit in the cancer cells, checkpoint activity declined and cells became sensitive to DNA damage and underwent apoptosis.
1.6 Conclusion

These findings suggest that the FRA3B/FHIT locus is not just a fragile chromosome region that upon breakage serves to alert cells passively of replication defects. Instead, the collective evidence supports a model where the fragile site – encoded FHIT protein actively guards the preneoplastic genome by modulation of DNA damage checkpoints to prevent acquisition of further mutations and tumorigenic alterations. Accordingly, cells with FRA3B/FHIT deletions are selected for in the clonal expansion in the preneoplastic stages of cancer development (Figure 1.4). Finally, FHIT loss occurs in dysplastic lesions concurrent with the onset of genome instability. Because of the genome guardian function of FHIT, the central hypothesis of this thesis is that loss of FHIT expression initiates genome instability and the neoplastic process.
Figure 1.4. FRA3B instability and subsequent loss of FHIT expression impairs the DNA damage checkpoints and confers a selective advantage to precancer cells facilitating further cancerous changes.
Chapter 2. Initiation of Genome Instability and Preneoplastic Processes through Loss of FHIT Expression

(Published manuscript: Saldivar et al., PLoS Genetics. 2012; 8(11):e1003077)

2.1 Introduction

Genomic instability drives tumorigenesis by expediting the acquisition of mutations that provide for selective clonal expansion and escape of normal cellular restraints (Hanahan and Weinberg, 2011). Expressions of genome instability include chromosomal instability, microsatellite instability, and instabilities typified by an increased frequency of point mutations. Chromosome instability is the most commonly observed form of genome instability, occurs in the majority of sporadic cancers and includes structural chromosome aberration (translocations, inversions, deletions and duplications) or numerical abnormality (aneuploidy, triploidy, tetraploidy) (Negrini et al., 2010). Because of its occurrence in most cancers, the molecular basis of chromosome and genome instability has been the subject of intense investigation. Chromosome and genome instability terminology is used interchangeably in this study to refer to chromosome structural and numerical abnormalities.
In several familial cancer syndromes, genome instability develops due to inherited mutations in the “DNA caretaker” genes essential for DNA repair or the DNA damage response. However, in sporadic cancers the known DNA caretaker genes are rarely mutated before the rise of genome instability (Kinzler and Vogelstein, 1997). It has been proposed that in early stage of sporadic tumorigenesis, activated oncogenes induce replication stress through deregulation of cell cycle progression, causing chromosomal instability, first at common fragile sites, and later throughout the genome (Bartkova et al., 2005; Gorgoulis et al., 2005). This proposal was corroborated by a report that expression of activated oncogenes *in vitro* results in nucleotide pool levels inadequate to support normal DNA replication, due to premature S phase entry (Bester et al., 2011). Remarkably, exogenously supplied nucleosides suppressed oncogenesis in the model systems studied.

While oncogene activation can induce replication stress *in vitro* and in mouse models, it also activates DNA damage response checkpoints and causes cellular senescence, forming a barrier to cancer progression (Bartkova et al., 2006; Di Micco et al., 2006). Without inactivating mutations in DNA damage response genes or experimental manipulation of cell cycle checkpoints, transformation does not occur. This suggests that genome instability or mutational diversity in genetically un-manipulated models occurs prior to oncogene activation. Moreover, recent studies have detected the presence of clonal somatic mosaicism in a small fraction of healthy individuals. These chromosomal anomalies are more prevalent in older individuals, and precede oncogenesis (Jacobs et al., 2012; Laurie et al., 2012), in accord with the idea that genome instability
initiates prior to oncogene activation. It has also been argued that since cancers with microsatellite instability, but not chromosomal instability, similarly express the activated oncogenes proposed to induce chromosomal instability, such oncogenes do not actually cause chromosome alterations (Cahill et al., 1999). Finally, genomic alterations are observed in human precancerous lesions, yet there are few reports of activated oncogenes in such lesions. Thus, oncogene-induced DNA damage contributes to the progression of genome instability in sporadic cancers, but is unlikely to initiate it.

Deletions at common fragile site FRA3B do occur in preneoplasias and may be the most frequent and earliest alterations. FRA3B overlaps the FHit gene, and FRA3B fragility often results in deletions of FHit exons and loss of FHit expression in precancer and cancer cells (Sozzi et al., 1998). Paradoxically, examination of cells that have lost the FHit gene product has revealed that FHit protein has functional roles in response to DNA damage (Saldivar et al., 2010): 1) kidney epithelial cells established from Fhit−/− mice exhibited >2-fold increased chromosome breaks at fragile sites vs corresponding Ffhit+/+ kidney cells (Turner et al., 2002); 2) the frequency of mutations following replicative or oxidative stress in FHit-deficient transformed and cancer cells was 2 to 5-fold greater than in FHit-expressing cells (Ishii et al., 2008; Ottey et al., 2004). Despite these findings and strong evidence that FHit exerts tumor suppressor activity (Joannes et al., 2010; Siprashvili et al., 1997), it has been argued that deletions within the FHit locus in transformed cells are passenger alterations rather than cancer-driving mutations (Bignell et al., 2010). In this study we have further examined the role of FHit loss in development of DNA damage and observed that absence of FHit causes
genome instability without activating the DNA damage response and senescence barrier. Our findings support a model for the initiation of genome instability in early stages of neoplasia through $FHIT$/FRA3B alterations and subsequent loss of FHIT function.

2.2 Materials and Methods

Ethics Statement

The experiments involving isolation of mouse tissues for DNA analysis and for establishment of cell lines were done according to a protocol approved by the Ohio State University Institutional Animal Care and Use Committee (IACUC).

Cell lines and reagents

HEK293 cells and $Fhit^{+/+}$ and $Fhit^{-/-}$ mouse kidney cells from C57Bl/B6 background mice, were cultured in MEM with 10% FBS and 100 µg/ml gentamicin. FHIT-deficient H1299 lung carcinoma cells were previously transfected with an inducible $FHIT$ cDNA and tightly regulated inducible clones were isolated, including the D1 clone; empty vector control clones, including E1 cells, were also isolated (Cantor et al., 2007). For experiments using E1 and D1 cells, FHIT expression was induced by addition of ponasterone A (ponA) to growth medium (MEM, 10% FBS, gentamicin, zeocin and geneticin) for 48 h. For certain experiments, 2 mM hydroxyurea (Sigma) was added to cells for times indicated in text.

siRNA transfections

HEK293 cells (60-80% confluent) were transfected with siRNAs targeting human $FHIT$ or a non-specific control siRNA (Santa Cruz Biotechnology) using the manufacturer's recommended protocol. For each 60 mm$^2$ dish, 1 µg of siRNAs and 5 µl of Lipofectamine
2000 (Invitrogen) were diluted in Opti-MEM (Gibco) and incubated for 30 min. Cells were washed in Opti-MEM, overlaid with the siRNA/Lipofectamine solution and incubated overnight at 37°C. Verification of siRNA knockdown of FHIT expression was performed 48-96 h later by Western blot.

**Comet assays**

Neutral comet assays were performed using the CometAssay kit (Trevigen) and recommended protocol. Images were acquired with a Zeiss Axioskop 40 fluorescent microscope mounted with an AxioCam HRc camera, and using an A-Plan 10x/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), fixed with 4% paraformaldehyde, permeabilized with ice-cold 70% ethanol and blocked in 1% BSA. Primary antisera, rabbit anti-\(\gamma\)H2AX, 1:200 (Cell Signaling Technologies); rabbit anti-53BP1, 1:200 (Cell Signaling Technologies); rabbit anti-pATR, 1:100 (Cell Signaling Technologies); mouse anti-Cyclin A, 1:100 (Santa Cruz Biotechnology); mouse anti-BrdU, 1:100 (Millipore), were added and cells incubated with antisera overnight at 4°C. Slides were washed 3 x 10 min in PBS, and secondary antisera (AlexaFluor 488 or 594 - conjugated donkey anti-rabbit IgG or anti-mouse IgG, 1:500, Molecular Probes) were 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. Brightness and contrast were adjusted equally for all images using Adobe Photoshop, and images were analyzed using Image J software.

**Western blot analysis**

Cells were lysed with RIPA buffer (Thermo Scientific) supplemented with Halt Protease Cocktail Inhibitors (Thermo Scientific). Proteins were separated by SDS gel electrophoresis, transferred to nylon membranes and immunoblotted with antisera against human FHIT (Pichiorri et al., 2009), GAPDH (Calbiochem), or phospho-Chk1 (Ser 317) (Cell Signaling).

**Flow cytometry**

siRNA transfected HEK293 cells were prepared for flow cytometric analysis of DNA content 72 h after transfection. Cells were harvested, fixed in ice-cold 70% ethanol at 4°C overnight. Cells were stained with propidium iodide solution (0.1 mg/ml propidium iodide, 0.1% Triton X-100, 0.2 µg/ml DNase-free RNase A) for 30 min and analyzed using a BD FACS Calibur.

**DNA fiber analysis**

Cells were pulsed with 25 µM CldU for 30 min, washed, and pulsed with 250 µM IdU for 30 min. DNA fibers were prepared as described (Jackson and Pombo, 1998). Cells were resuspended in PBS at 10^6 cells/ml, 2 µl were spotted on glass sides and lysed with 5 µl of lysis buffer (0.5% SDS, 200 mM Tris-HCl, pH 7.4, 50 mM EDTA) for 10 min. Slides were tilted 15° to stretch DNA fibers by gravitational flow. Fibers were fixed with methanol/acetic acid (3:1), denatured with 2.5 N HCl for 1 h, and blocked with 1% BSA.
Rat anti-BrdU (1:50, AbD Serotec) was used to detect CldU, and mouse anti-BrdU (1:20, Becton Dickinson) to detect IdU. Primary antibodies were fixed with 4% paraformaldehyde and detected with AlexaFluor 594 – conjugated donkey anti-rat IgG (1:250, Molecular Probes) and AlexaFluor 488 – conjugated donkey anti-mouse IgG (1:250, Molecular Probes). Coverslips were mounted using Fluoro-Gel II – with Dapi (Electron Microscope Sciences). Images were acquired at room temperature using an Olympus FV1000 spectral confocal microscope and with a PLAPON 60XO objective lens, NA 1.42. Fiber lengths were measured using Olympus FLUOVIEW software, and velocities were estimated using a conversion factor of 1 µM = 2.59 kbp. Brightness and contrast were adjusted equally for all images using Adobe Photoshop.

**Preparation of metaphase spreads**

Metaphase chromosomes were prepared from Fhit+/+ or Fhit−/− mouse kidney cells at the 8th subculture. Cells were treated with colcemid (0.1 µg/ml) for 1 h to block cells in mitosis. Cells were trypsinized, pelleted and resuspended in 0.075M KCl hypotonic solution for 10 min at 37°C. Cells were fixed in methanol/acetic acid (3:1), dropped on glass slides and allowed to air dry. Chromosomes were analyzed using a Zeiss Axioskop Widefield LM at 100X magnification.

**3T3 cell culture and analysis of cell growth kinetics**

Mouse embryonic fibroblasts were isolated from individual 13-day embryos of Fhit+/+ and Fhit−/− pregnant females and cultured in Dulbecco Modified Eagle’s Medium with 10% fetal bovine serum and 100 µg/ml gentamicin. Primary MEFs were subcultured by trypsinizing and replating 3 x 10^5 cells per 6 cm² dish every three days (3T3 protocol).
The time of immortalization was defined retrospectively as the first tissue culture passage after which the cell population increased consistently with each subculture.

**Copy number variation analysis**

Genomic DNA was isolated from $Fhit^{+/+}$ and $Fhit^{+/−}$ MEFs at tissue culture passages 3 and 25 (n = 3 lines, 1 from each of 3 embryos, for each pair at each passage) using DNeasy Blood and Tissue (Qiagen). Genomic DNA was also isolated from $Fhit^{+/+}$ and $Fhit^{+/−}$ weanling tail DNA. Genomic DNA samples were analyzed for copy number aberrations at Jackson Labs using the Affymetrix Mouse Diversity Genotype Array. $Fhit^{+/+}$ tail DNA served as reference DNA.

**Statistical analysis**

For all boxplots, bottom and top of the box correspond to the 25th and 75th percentiles, respectively, and whiskers represent data points within 1.5 x IQR (interquartile range). The gray line extending through the boxplot indicates the mean value, and the black line contained within the boxplot represents the median value. Two-sided T-tests were used to determine significance for data with a normal distribution and equal variances. Nonparametric data was analyzed using the Mann-Whitney rank sum test for single comparisons or using the Kruskal-Wallis test for multiple comparisons. Groups with P-values less than an alpha of 0.05 were considered significantly different.

**2.3 Results**

**2.3.1 FHit-deficient cells exhibit spontaneous DNA breaks**

To define the role of FHit in promoting genome stability, we began by assessing spontaneous DNA damage in 3 different cell line models: transformed HEK293 cells
(FHIT-positive), H1299 lung adenocarcinoma cells (FHIT-negative), and primary kidney epithelial cells established from \textit{Fhit}^{+/+} and \textit{Fhit}^{-/-} mice. We chose these 3 cell lines to model \textit{in vitro} different stages of tumorigenesis, from normal to cancerous. HEK293 embryonic kidney cells were transfected with siRNAs targeting \textit{FHIT} to transiently silence FHIT protein expression (Figure 2.1A). FHIT-negative H1299 lung adenocarcinoma cell clones carry either an inducible \textit{FHIT} cDNA expression plasmid (D1 clone) or the empty vector control (E1 clone). Addition of ponasterone A (PonA) to culture medium robustly induces FHIT expression in the D1 clone but not in the E1 clone (Figure 2.1B). DNA damage was measured by neutral comet assay, or single cell gel electrophoresis assay, a method routinely used to detect DNA double-strand breaks (DSBs). The assay is based on the principle that fragmented DNA migrates faster than un-fragmented DNA through agarose gel in an electric field. Undamaged DNA remains in the nucleoid and is seen as the “comet head”, while damaged DNA migrates through the gel and forms the “comet tail” (Figure 2.2A). We used the tail moment, the product of tail length and % of total DNA in the tail, to score DSB levels in individual cells.
Two days following FHit knockdown, we observed a significant increase in the mean tail moment in FHit-deficient cells vs si-control cells, indicating that decreased FHit expression resulted in spontaneous DSBs. Co-transfection with FHit siRNAs and a FHit expression plasmid suppressed DSB formation, confirming that FHit-depletion caused the DSBs (Figure 2.2B). As independent confirmation of DSBs following FHit knockdown, we assessed numbers of nuclear γH2AX and 53BP1 foci, markers of DNA breaks, by indirect immunofluorescence (Figure 2.2C). HEK293 cells transfected with FHit siRNAs exhibited a ~3-fold increase in the fraction of cells with γH2AX and 53BP1 foci (Figure 2.2D and E), confirming that loss of FHit expression causes DSBs.

To determine if FHit prevents DSBs in normal cells, we compared tail moments in primary cells established from Fhit¬/¬ mouse kidney vs cells from wild-type Fhit+/+ kidney. The mean tail moment of Fhit¬/¬ cells was ~2-fold greater than that of Fhit+/+ cells and exogenous FHit expression in the Fhit¬/¬ cells decreased the mean tail moment.
(Figure 2.2F). After induction of FHIT expression in D1 cells, the mean tail moment was significantly reduced (Figure 2.2G). Collectively, these results demonstrate the critical role for FHIT in suppressing spontaneous DSBs in transformed cells and cells derived from normal and cancerous tissue.
Figure 2.2. FHIT-deficient cells exhibit spontaneous DNA breaks. (A and B) Neutral comet assays of HEK293 cells 2 days after transfections with siRNAs and pRcCMV-FHIT-flag or empty plasmids; bars, 20 µm. Box plots of Tail moments: siCtrl, n = 183; siFHIT, n = 142; siFHIT + CMV-ev, n = 135; siFHIT + CMV-FHIT, n = 132. (C) Indirect immunofluorescence of γH2AX and 53BP1; bars, 10 µm. (D and E) Quantification of γH2AX-positive cells (D) and 53BP1-positive cells (E). P-values were calculated using a 2-sided T-test. (F and G) Neutral comet assays of Fhit+/+ or Fhit−/− mouse kidney cells 48 h after transfection with pRcCMV-FHIT-flag or empty plasmids (F) or H1299 cells with or without ponA (G). P-values were calculated using the Mann-Whitney rank sum test.
2.3.2 FHit prevents endogenous replication stress and replication fork stalling

Since endogenous DSBs typically form due to DNA replication defects (Aguilera and Gómez-González, 2008), we asked if FHit-deficient cells exhibit increased replication stress. We co-immunostained FHit-silenced HEK293 cells with antisera against γH2AX and Cyclin A, an S/G2 phase marker. FHit-deficient cells exhibited a dramatic increase in γH2AX immunofluorescence staining in Cyclin A-positive cells (Figure 2.3A and B), suggesting that DNA breaks were caused by aberrant DNA replication. Similar results were obtained using H1299 cells, as induction of FHit expression in D1 cells led to reduced numbers of γH2AX foci in Cyclin A-positive cells (data not shown). To determine if DNA damage occurred specifically at sites of replication, we immunostained for phospho-ATR (Ser428), a kinase that localizes to stalled replication forks and initiates the S phase checkpoint (Cimprich and Cortez, 2008). FHit knockdown resulted in a ~3-fold increase in the fraction of cells with phospho-ATR (Ser428) nuclear foci vs HEK293 cells treated with control siRNAs (Figure 2.3C and D). To verify that replication forks were more frequently damaged in FHit-deficient cells, we pulse-labeled H1299 E1 and D1 cell replication forks with BrdU, and immunostained for γH2AX and BrdU. In these cells, induction of FHit expression in D1 cells led to reduced γH2AX localization to BrdU-labeled replication foci (data not shown). We conclude that FHit functions to decrease DNA damage arising from endogenous replication stress.

We reasoned that FHit may either function upstream to prevent and minimize DNA replication stress or, alternatively may contribute to downstream replication fork maintenance and thereby prevent fork collapse and DSB formation. To distinguish
between a role of FHIT upstream or downstream of replication stress, we treated cells
with hydroxyurea (HU) for 4 h and measured comet tail moments. HU causes replication
fork stalling through depletion of dNTPs by inhibition of ribonucleotide reductase; thus
cells treated with HU for more than 2 h accumulate inactivated replication forks and
DSBs (Petermann et al., 2010a; Sirbu et al., 2011). If FHIT functions to support
replication fork stability after replication stress, then HU challenge should induce more
DSBs in FHIT-deficient cells. However, HU-treated FHIT-silenced HEK293 cells and
treated control cells exhibited similar levels of comet tail moments (Figure 2.3E). Similar
results were obtained in H1299 D1 and E1 cells (Figure 2.3F). Overall, HU treatment
resulted in equivalent tail moments in FHIT-expressing and FHIT-deficient cells,
suggesting that FHIT does not function downstream of replication stress.

Next we investigated the possibility that FHIT supports normal DNA replication,
such that silencing FHIT causes replication stress, through analysis of DNA replication
dynamics at the single-molecule level (Jackson and Pombo, 1998). DNA fibers from
HEK293 cells pulsed sequentially 30 min each with the nucleotide analogs, 5-
chlorodeoxyuridine (CldU) and 5-iododeoxyuridine (IdU), were spread on glass slides.
Figure 2.3. Loss of FHIT causes replication stress. (A and B) Cyclin A and γH2AX indirect immunofluorescence after FHIT knockdown; bars, 10 µm. (C and D) pATR immunofluorescence 2 days after FHIT knockdown in HEK293 cells; bars, 5 µm. (A-D) Three independent experiments were performed and statistical significance was determined using a 2-sided T-test. (E and F) Neutral comet assays in siRNA transfected HEK293 cells (E) or H1299 E1 and D1 cells with ponA-induction (F) treated with 2 mM HU for 4 h. P-values were determined using the Mann-Whitney rank sum test.
Replicating DNA incorporates CldU and then IdU during the sequential pulses, and is detected by immunofluorescence (Figure 2.4A). Labeled DNA fibers were consistently shorter in cells transfected with FHIT siRNAs vs control siRNAs. Using a conversion factor of 1 µm = 2.59 kilobase pairs (kbp) (Jackson and Pombo, 1998), average fork velocities of 1.05 kbp/min were estimated for control and 0.61 kbp/min for FHIT-silenced cells (Figure 2.4B). The results illustrate the role of FHIT in sustaining normal DNA replication.

We also assessed the symmetry of sister replication forks proceeding outward from common replication origins that fired during the CldU pulse (Figure 2.4C). Because DNA synthesis is coupled at sister replication forks, asymmetrical DNA synthesis is thought to represent stalling or collapse of one of the forks (Conti et al., 2007). Control cells exhibited mostly symmetrical sister forks, with length of IdU-labeled tracts on either side of the replication origin nearly equal. In contrast, FHIT knockdown increased the frequency of asymmetrical sister forks proceeding outward from a common origin (Figure 2.4C-E), suggesting that FHIT loss results in increased fork stalling and collapse. We also assessed replication dynamics in mouse kidney cells and H1299 cancer cells; replication defects were also observed in Fhit⁻/⁻ mouse kidney cells and FHIT-deficient H1299 cells (Figure 2.4F and G). Overall, the results suggest that FHIT does not participate in the response to fork stress, but rather, in unperturbed conditions FHIT promotes normal DNA replication progression.
Figure 2.4. Loss of FHIT expression causes fork stalling. (A and B) Fork velocities of siRNA transfected HEK293 cells; bars in A, 10 µm. Statistical significance was determined using a 2-sided T-test (n = 238 for siCtrl; n = 320 for siFHIT). (C-E) Sister fork symmetry in siRNA transfected HEK293 cells; bar in C, 10 µm. (D) Scatter plots of distances traveled by left and right sister forks. The central area marked by red lines represents sister forks with less than 25% length difference. The percentages of asymmetric sister forks are indicated at the upper left region of plots. (E) Fork asymmetry is calculated as the ratio of the longest IdU tract to the shortest for each pair of sister forks. P-value was determined using the Mann-Whitney rank sum test. (F and G) Fork velocity in Fhit+/+, Fhit−/− and Fhit−/− pReCMV-FHIT-flag plasmid transfected mouse kidney cells (F) or in H1299 E1 and D1 cells with ponA (G).
2.3.3 Genomic instability induced by FHit-deficiency does not activate the S/G2 checkpoint

DSBs are the most deleterious DNA lesions as they are difficult to repair correctly and often lead to complex genomic alterations, including large deletions, duplications, and translocations (Aguilera and Gómez-González, 2008). Non-random mutation clusters can also arise during repair of DSBs or following restart of collapse replication forks (Deem et al., 2011; Roberts et al., 2012). Cells minimize replication stress-induced DSB formation by activating the S phase replication checkpoint to block cell cycle progression and coordinate replication fork stabilization and restart (Cimprich and Cortez, 2008). Central to the S phase checkpoint are the kinases ATR and Chk1. ATR localizes to stalled forks and phosphorylates multiple targets, including Chk1. Phosphorylated Chk1 is then activated to phosphorylate its targets, setting off a cascade of events to enforce the S phase checkpoint. We investigated the checkpoint response to FHit-silencing in HEK293 cells, as these cells exhibit replication defects and spontaneous DSBs, first by immunoblot of lysates of HEK293 cells transfected with FHit siRNAs, to assess expression of phospho-Chk1 (Ser317). We did not detect increased expression of phospho-Chk1 following FHit knockdown, suggesting that the S phase checkpoint was not activated (Figure 2.5A). We also assessed the cell cycle distribution by flow cytometric analysis of DNA content to determine if FHit-deficient cells accumulated in S or G2 phase. Consistent with the phospho-Chk1 western blot data, FHit knockdown did not cause accumulation of cells in S or G2 phase (Figure 2.5B and C).
While surprising, the results are not without precedent. For example, it is known that cells can traverse mitosis with under-replicated DNA due to replication stress and subsequently form DNA lesions marked by 53BP1 nuclear bodies in daughter G1 cells.
(Harrigan et al., 2011; Lukas et al., 2011). Thus, we determined if FHIT-silenced cells complete cell division despite endogenous replication stress and DNA damage, by assessing the incidence of 53BP1 nuclear bodies in G1 cells by immunofluorescence staining of 53BP1 and Cyclin A. Within 3 days, FHIT knockdown in HEK293 cells led to a significant increase in the number of 53BP1 bodies in G1 phase cells, defined as the Cyclin A-negative cells (Figure 2.6A and B). Prolonged knockdown of FHIT in HEK293 cells, by transfecting fresh FHIT siRNAs every 4 days for 2 weeks, led to an even greater incidence of 53BP1 foci per G1 phase cell (Figure 2.6C). The results suggest that FHIT-deficient cells continue to proliferate and accumulate replication stress-induced DNA alterations.

In addition to the 53BP1-marked lesions, cells can acquire other replication stress-induced chromosomal aberrations during mitosis. For example, replication stress can cause micronucleus formation, due to failed segregation of chromosome fragments broken at fragile sites during mitosis or due to nondisjunction of chromosomes with incompletely replicated loci or unresolved replication intermediates (Chan et al., 2009; Naim and Rosselli, 2009; Xu et al., 2011), events that result in large deletions or aneuploidy, respectively. In FHIT-silenced HEK293 cells, we observed an ~3-fold increase in the percent of micronucleated cells relative to control cells (Figure 2.6D and E), confirming that replication stress caused chromosomal alterations. To measure aneuploidy incidence, we analyzed metaphase spreads from normal kidney cells established from Fhit
\textsuperscript{+/+} and Fhit
\textsuperscript{−/−} mice. At passage 8, nearly 30% of Fhit
\textsuperscript{−/−} cells were aneuploid, compared to fewer than 5% of the Fhit
\textsuperscript{+/+} cells (Figure 2.6F). In addition,
Fhit−/− cells exhibited a 2-fold increase in the number of chromosome breaks/metaphase (Figure 2.6G). Taken together, the results show that Fhit loss–induced replication stress causes DNA lesions and chromosomal abnormalities following cell division in the absence of DNA damage checkpoint activation.
Figure 2.6. Loss of FHIT causes replication stress-induced chromosomal instability. (A-C) Cyclin A and 53BP1 immunofluorescence after FHIT knockdown in HEK293 cells; bar in A, 20 µm. Histograms of 53BP1 nuclear bodies/G1 phase cell 3 days (B) or 14 days after siRNA transfection (C). Mann-Whitney rank sum test was used to determine P-values. (D and E) Micronucleus formation 3 days after siRNA transfections. Arrow marks a micronucleus; bar in D, 5 µm. (F and G) Percentage of aneuploid and tetraploid cells established from $Fhit^{+/+}$ or $Fhit^{-/-}$ mouse kidney (F) and frequency of breaks/metaphase (G); (n = 37 for $Fhit^{+/+}$; n = 40 for $Fhit^{-/-}$ metaphases).
2.3.4 Genomic alterations caused by FHIT loss expedite cell immortalization

Because *FHIT* is an early target of allelic deletion in preneoplasia (Bartkova et al., 2005; Gorgouli et al., 2005; Tsantoulis et al., 2008) and loss of FHIT protein expression induces replication stress, micronucleation and aneuploidy, we determined if FHIT-deficiency contributes to the onset of genomic instability in cells undergoing immortalization *in vitro*. Mouse embryo fibroblasts (MEFs) were established from *Fhit<sup>+/+</sup>* and *Fhit<sup>-/-</sup>* embryos (3 embryos per mouse strain) and were immortalized using the 3T3 protocol. *Fhit<sup>-/-</sup>* MEFs became immortalized and exhibited rapid growth at earlier tissue subcultures (passage 12, 14, and 16) compared to matching *Fhit<sup>+/+</sup>* MEFs (passage 14, 20, and 20) (Figure 2.7A). Fhit expression in *Fhit<sup>+/+</sup>* MEFs decreased as cells became immortalized (Figure 2.7B), and for the *Fhit<sup>+/+</sup>* MEF cell line showing rapid growth and immortalization by passage 14, a corresponding early loss of Fhit expression occurred. Therefore, loss of Fhit expression may be selected for as an essential step in the process of immortalization.

We then assessed somatic copy number aberrations, defined as DNA amplifications or deletions spanning more than 10 kb in size, using genomic DNA isolated from *Fhit<sup>+/+</sup>* and *Fhit<sup>-/-</sup>* MEFs grown in culture, pre- and post-senescence (at subcultures 3 and 25). Multiple copy number aberrations were detected in the pre- and post-senescence *Fhit<sup>-/-</sup>* MEFs, whereas only one was observed in one *Fhit<sup>+/+</sup>* MEF line (Figure 2.7C and Table A.1). Somatic aberrations in the *Fhit<sup>-/-</sup>* MEFs occurred at 8 different genomic loci, 6 of which corresponded with fragile sites previously mapped in mouse fibroblasts (Djalali et al., 1987) or lymphocytes (Helmrich et al., 2006) (Table...
A.2), which implies that the genomic changes were caused by replication stress, and is consistent with reports that replication stress induces copy number changes (Arlt et al., 2009; Arlt et al., 2011). Across the 3 Fhit−/− MEF lines analyzed, 12 somatic aberrations occurred only in post-senescent cells, suggesting clonal expansion of cells harboring these genomic changes. Notably, 2 of the 3 Fhit−/− MEF cell lines acquired allelic gains within chromosome band 10D2, encompassing the murine Mdm2 gene, an oncogene involved in cell transformation, and Mdm2 gene amplification correlated with ~4-fold increase in Mdm2 mRNA expression. These amplifications were likely selected for during immortalization, as they were present only in the cells that had escaped senescence.

Copy number aberrations were also observed in genomic DNA from Fhit−/− weanling tail tissue (Table A.3). Most Fhit−/− tail DNA aberrations did not overlap with those observed in MEF cultures, suggesting that genomic instability is prevalent in Fhit−/− tissue early during development and copy number aberrations are selected during in vitro growth, depending on environment (eg, pre-senescent, senescent and post-senescent cultures). Genome instability has previously been observed in p53- and Gadd45a-deficient mice, where aneuploidy is detected in primary cells from multiple tissues (Fukasawa et al., 1996; Fukasawa et al., 1997; Hollander et al., 1999). Unlike the chromosomal instability in p53- and Gadd45a-deficient mouse cells, which exhibit aneuploidy due to centrosome amplification and mitotic errors, Fhit-deficient mice exhibit signs mostly of replication stress-induced DNA deletions and gains. Based on these observations and the fact that the genomic alterations begin in the knockout mouse
tissue, we propose that the deletions within *FHIT* loci observed in preneoplastic human tissues *in vivo* initiate genomic instability and accelerate the neoplastic process.

**Figure 2.7** Genomic instability in Fhit-deficient cells correlates with onset of rapid proliferation and immortalization. (A) Analysis of *Fhit<sup>+/+</sup>* and *Fhit<sup>−/−</sup>* 3T3 MEF cell lines (*n* = 3, cell lines established from 3 embryos for each mouse strain). Arrows mark the passage numbers when MEFs became immortalized. (B) Western blot of *Fhit<sup>+/+</sup>* MEFs for Fhit and GAPDH expression. Immunoblots were performed on lysates obtained at the indicated passage number. (C) Summary of copy number aberrations (CNAs) in pre- and post-senescence MEFs from *Fhit<sup>+/+</sup>* and *Fhit<sup>−/−</sup>* mice.
2.4 Discussion

2.4.1 Model for FHIT loss-induced genome instability

This study has shown that loss of FHIT expression promotes the development of genomic instability. Consequently, loss of FHIT expression indirectly impairs replication fork progression, leading to fork stalling and DNA double-strand breaks. In FHIT-deficient cells, replication defects fail to activate the S or G2 checkpoints, and as cells complete mitosis, chromosomal alterations occur and are propagated to daughter cells. This process continues with each cell division cycle, and chromosomal instability inevitably arises. Upon acquisition of oncogenic mutations, selective pressures expedite cell transformation (see proposed model in Figure 2.8).

![Diagram](image)

**Figure 2.8. The FHIT loss–induced genome instability model.** Deletions in *FHIT* alleles occur due to FRA3B fragility causing loss of FHIT protein expression. FHIT loss triggers replication stress, followed by stress-induced chromosomal instability. Chromosomal instability increases the likelihood of activating mutations in oncogenes and/or inactivating mutations in tumor suppressors, which are then selected for, facilitating cell transformation.
Several alternative models have been proposed as common mechanisms for the origin of genome instability, including oxidative stress, telomere erosion, impaired DNA repair and chromosome segregation errors; however, these forms likely do not contribute to the initiation of instability but rather to ongoing instability as they are seen in more advanced lesions (Halazonetis et al., 2008). The prevailing hypothesis for the origin of genome instability in preneoplastic cells is that defects in DNA replication result in DNA breaks and when incorrectly repaired, they produce chromosomal changes (Aguilera and Gómez-González, 2008). Therefore, it is important to define the molecular source of replication stress that initiates genome instability. Oncogene activation can cause replication stress, chromosomal instability and promote tumorigenesis, and has been proposed as a mechanistic basis of genome instability (Negrini et al., 2010). However, oncogene-induced replication stress is probably not the initiating event. For example, oncogene activation is achieved through various mechanisms that involve chromosome alterations, including translocations that change expression of the oncogene, duplications that increase the oncogene copy number, point mutations within the oncogene that increase its activity, deletions of a negative regulator, or epigenetic changes that affect gene expression. Also, because many oncogenes that induce senescence require a second genetic “hit” to uncouple mitogenic signaling from the senescence barrier (Lowe et al., 2004), it seems probable that some degree of genetic instability and heterogeneity must precede oncogene activation.

There are two important distinctions in the FHIT-loss model that make it a more likely mechanism for the origin of genome instability. First, because of the inherent
fragility at the FRA3B locus, the FHit gene has been called the “weakest link” in the genome (Huebner and Croce, 2001), making it a first target for inactivation in cells undergoing transformation, and its deletion a strong candidate initiator of genomic instability. Indeed, alterations at the FHit/FRA3B locus are occasionally detected in normal cells without exposure to known inducing agent. These alterations can be caused by normal metabolic processes or by exposure to thus far undefined environmental stresses. The second important feature of the FHit loss-induced genomic instability model is that cells acquire replication stress-induced chromosomal alterations without DNA damage response activation, in contrast to observations in oncogene-activated cells, possibly because the replication defects caused by FHit loss fall below the threshold level needed to fully activate the S and G2 checkpoints. Likewise, aphidicolin induces fragile site expression by slowing or stalling replication forks, yet fragile sites are routinely detected in metaphase chromosomes, indicating a failure of the S and G2 checkpoints to block mitotic entry despite the presence of damaged loci. Furthermore, studies have suggested that eukaryotic cells lack a checkpoint surveillance mechanism to insure completion of DNA replication before mitotic entry (Torres-Rosell et al., 2007). Thus, it is possible that DNA replication is incomplete in FHit-deficient cells because of slower fork progression, and as cells pass through mitosis, under-replicated chromosomes either break or fail to properly segregate. In theory, without DNA damage checkpoint activation, FHit-deficient cells could continue to proliferate for years, and over time accumulate extensive genome alterations generating significant mutational diversity and cell heterogeneity, as is the case with Fhit−/− mice. Indeed, Fhit−/− mice develop normally
and live long lives, making \textit{FHIT} inactivation an ideal target to initiate genome instability without compromising fitness at the cellular and organism levels. Thus, \textit{FHIT} loss would provide the “soil” for the emergence of preneoplastic clones under selective pressure. This is consistent with the recent finding that clonal somatic chromosome anomalies increase with age in the normal population (Jacobs et al., 2012; Laurie et al., 2012) and is consistent with the fact that cancer risk increases with age. It is also consistent with the enhanced susceptibility of \textit{Fhit}−/− mice to development of spontaneous hyperplastic lesions and tumors and their highly enhanced susceptibility to carcinogen-induced tumors (Zanesi et al., 2001).

\textbf{2.4.2 \textit{FHIT} deletion as a cancer-driving mutation}

Our findings strongly support the view that loss of \textit{FHIT} provides a selective advantage in sporadic cancers, directly or indirectly, because \textit{FHIT}-deficient cells, which are genomically unstable, have a greater likelihood of acquiring cancer-promoting mutations. The relevance of \textit{FHIT} loss during the neoplastic process has been inferred from the ~50% frequency of \textit{FHIT} loss in epithelial cancers (Huebner and Croce, 2001), and from its tumor suppressor activity. The demonstration that \textit{Fhit}−/− MEFs rapidly become immortalized and begin to acquire oncogenic DNA copy number aberrations, provides direct evidence of a genome ‘caretaker’ function for \textit{FHIT} that is lost early in tumorigenesis. The development of sebaceous gland tumors in \textit{Fhit}-deficient mice (Fong et al., 2000), a condition analogous to the sebaceous tumors of \textit{Muir-Torre} syndrome in mismatch repair-deficient mice and humans (Kruse et al., 1998; Reitmair et al., 1996), and the observation that there are two forms of sebaceous tumors in humans, one form
exhibiting mismatch repair gene deficiency and one exhibiting FHIT-deficiency (Becker et al., 2008; Goldberg et al., 2006), can now be understood as a classic illustration of the caretaker function of FHIT. We conclude that FHIT loss is a common underlying initiator of genome instability in preneoplasia and a driver of the transformation process.
Chapter 3. FHIT Supports DNA Replication through Nucleotide Salvage

3.1 Introduction

Replication stress is increasingly recognized as the major source of genome instability in sporadic cancer (Negrini et al., 2010). Replication stress often leads to replication fork collapse into DNA breaks (Petermann et al., 2010a) and when misrepaired produces chromosome structural changes (Malkova and Haber, 2012). Structural changes creating acentric or dicentric chromosomes cause segregation errors during mitosis and lead to chromosome numerical changes in daughter cells (Burrell et al., 2013). Mechanisms that restart collapsed replication forks are error-prone and insert clusters of small insertions and deletions and single-nucleotide substitutions (Iraqui et al., 2012). Methylated cytosines within ssDNA formed at stalled replication forks are targeted by the APOBEC cytosine deaminases producing C to T transitions and are a major source of mutations in breast cancer (Burns et al., 2013; Nik-Zainal et al., 2012; Roberts et al., 2012). Notably, genome instability can be dramatically reduced in cell culture models by alleviating the source of replication stress (Bester et al., 2011; Mannava et al., 2013). These discoveries highlight the central role of replication stress in generating genome instability.
One of the causes of replication stress is premature entry into S-phase as cells are unable to prepare sufficiently for the complex task of accurately duplicating the approximately 6.4 billion nucleotides of DNA (Bester et al., 2011). To replicate DNA during S-phase, cells must up-regulate the expression of several S-phase-specific proteins that perform essential functions in energy metabolism, nucleotide biosynthesis, replisome assembly, controlled origin firing, DNA synthesis, checkpoint surveillance, cell cycle progression and regulation. Premature S-phase entry may result in failure to perform one or more of these processes properly, and the consequent induction of replication stress (Robinson et al., 2009). The transition from G1- to S-phase is coordinated by transcription factors under the control of the cell cycle cyclins and associated cyclin-dependent kinases (Cdks) (Johnson et al., 1993). In G1-phase, cyclin D-Cdk4/6 complexes activate the E2F family of transcription factors that regulate many S-phase-specific genes, including transcription of cyclin E that activates Cdk2 to start S-phase (Ohtani et al., 1995).

The finding that FHIT-deficient cells exhibit spontaneous replication stress suggests that FHIT may control or coordinate an important process or processes in preparation for S-phase. Specifically, FHIT-deficient cells undergo an inefficient S-phase with reduced replication fork velocities, an indication of slower DNA synthesis kinetics, increased fork collapse and DNA damage (Saldivar et al., 2012). Nucleotide deficiency causes similar DNA replication defects during S-phase (Bester et al., 2011; Sánchez et al., 2012), and thus, the potential role of FHIT in regulating nucleotide production during S-phase was investigated.
3.2 Materials and Methods

Some experiments and cell lines used are described in detail in Chapter 2, including HEK293 cells, siRNA transfections, comet assays, western blots and DNA fiber assays.

Cell lines and reagents

A549 lung carcinoma cells with integration of a lentivirus containing shCtrl or shFHIT shRNAs were cultured in DMEM with 10% FBS, 100 µg/ml gentamicin and 1 µg/ml puromycin. HCT116 were cultured in DMEM supplemented with 10% FBS and gentamicin (100 µg/ml). For certain experiments, 10 µM thymidine (Sigma) was added to cells for times indicated in text. Immunoblots were performed using antisera against human TK1 (AbD serotec), mouse TK1, and human cyclin A (Santa Cruz Biotechnology).

dNTP pool measurements

HEK293 cells transfected with siFHIT or siCtrl were cultured in 10 cm² dishes for 72 h and dNTPs were extracted as previously described (Song et al., 2003). Exponentially growing cells were washed twice with ice-cold PBS, covered in ice-cold methanol and incubated at -20°C for 1-3 h. An additional plate of exponentially growing cells was used to calculate the total cell number per extract. Cell extracts were collected and incubated in boiling water for 3 min, and separated from cell debris by centrifugation at 17,000 x g, for 10 min at 4°C. dNTP extracts were dried using a Speed vacuum. dNTP pools were assayed using the enzymatic method. For each dNTP to be measured an oligonucleotide template was used (Mathews and Wheeler, 2009). The same primer was used for all four assays. The reaction mixture was mixed with either the dNTP extracts or known
standards used to construct a standard curve, and DNA polymerization was carried out at 37°C for 45 min. Reaction mixtures were spotted on DE81 chromatography paper, dried and washed in 5% Na$_2$HPO$_4$, followed by water, and finally in 95% ethanol. Samples were counted using a liquid scintillation counter. dNTP concentrations were determined by reference to the standard curve.

**Thymidine/nocodazole synchronization**

One day after siRNA transfections, cells were split 1:3 into 10 cm$^2$ plates. Ten hours later thymidine was added to the culture medium at a final concentration of 2 mM, and cells were incubated for 20 h. The culture medium was then removed, cells were washed 2x with PBS and normal growth medium was added. After 4 h of incubation in normal medium, nocodazole was added at a final concentration of 100 ng/ml, and cells were incubated for another 12 h. The culture medium was removed, cells were washed 2x with PBS and released into normal growth medium. Cells were collected every 4 h for up to 24 h and lysed for western blot analysis.

**RNA isolation and RT-qPCR analysis**

Total RNA was extracted from cells using the RNeasy kit following manufacturer’s recommendations (Qiagen). RNA reverse transcriptase reactions for cDNA synthesis were performed using Qiagen’s Quantitect Reverse Transcriptase reagents. qPCR was performed using BioRad’s SYBR Green mix on the IQ5 ICycler (BioRad). Gene expression-specific primers were purchased from Santa Cruz Biotechnology. PCR settings were as follows: 1 cycle of 95°C for 3 minutes; 35 cycles of 95°C for 15 seconds, 58°C for 30 seconds, and 70°C for 45 seconds. Threshold cycles (Ct) for each reaction
were normalized to GAPDH, ΔΔCt values were calculated, and mRNA expression was calculated relative to controls. For each independent experiment, qPCR reactions were performed in either triplicate or quadruplicate.

**RNA stability assay**

HEK293 cells were transfected with siRNAs and 3 days later actinomycin D was added to the medium at a final concentration of 10 μg/ml. RNA was extracted at 0, 2, 4, 8 and 12 h after actinomycin D challenge, and analyzed by RT-qPCR.

### 3.3 Results

#### 3.3.1 FHIT modulates the supply of dTTP

Genomic instability initiates following loss of FHIT expression, both in cultured human cell lines and in primary mouse tissue. The loss of FHIT expression in early precancerous cells is proposed to be a major contributor to the origin of genome instability seen in cancer cells (Saldivar et al., 2012). Specifically, FHIT-depletion causes a defect in replication fork progression and increases replication fork stalling and collapse, causing chromosomal alterations and instability with each cell division. To explore mechanisms involved in FHIT support of DNA replication progression, we considered the observation that depletion of dNTP pools by hydroxyurea exposure equalized DNA damage in FHIT-positive and deficient cells (see Chapter 2; Figure 2E and F). It is known that imbalance in dNTPs is mutagenic and leads to chromosomal abnormalities (Meuth, 1989), and in certain models, reduced dNTP pools aid transformation (Bester et al., 2011). Furthermore, the size of the dNTP pool controls replication fork progression (Poli et al., 2012). To determine if such imbalances occur
spontaneously following knockdown of FHIT expression, we measured dNTP pools using the enzymatic assay developed by Sherman and Fyfe (Sherman and Fyfe, 1989). Within 72 h, FHIT knockdown in HEK293 cells caused ~30% reduction in the dTTP level compared to control cells, with other dNTPs unaffected (Figure 3.1A). In these experiments FHIT knockdown relative to control cells ranged from 35-75%; to account for differences in knockdown efficiency, we plotted the relative FHIT expression vs the relative dTTP levels, revealing that cells with greater knockdown of FHIT expression had lower levels of dTTP, up to 50% lower than control cells (Figure 3.1B). To determine if the effect on dTTP pools was a transient response to FHIT knockdown, we used A549 lung carcinoma cells that were engineered for stable silencing of FHIT expression. dNTPs were extracted from these cells after 7-9 weeks of stable FHIT-knockdown, and the dTTP pool was still significantly reduced relative to control cells (Figure 3.1C). While a 25-50% reduction in dTTP levels seems a modest change, it may suffice to hinder replication fork movement and cause DNA breaks, especially at loci sensitive to mild replication stress such as fragile sites.
Figure 3.1. FHIT modulates the dTTP pool level. (A) Deoxyribonucleotide triphosphate (dNTP) levels in HEK293 cells 72 h after FHIT knockdown. Bar graphs represent means of 4 independent experiments, and error bars denote the standard deviations. The P-values were determined using a 2-sided T test; ns = not significant. (B) Correlation of relative FHIT expression and relative dTTP levels. siRNA transfected HEK293 cells were split into matching pairs, one for dNTP analysis and the other for western blot analysis of FHIT knockdown. (C) dNTP measurements in A549 cells with 7-9 week stable FHIT knockdown. Bar graphs represent means of 7 independent experiments.

For example, low doses of hydroxyurea (0.1 mM) moderately reduce dNTP levels (by 20-40%) yet fully block DNA replication (Bianchi et al., 1986). Moreover, treatment with 0.05 mM hydroxyurea significantly decreased replication fork movement and caused an increase in DNA breaks (Figure 3.2A-C).
Figure 3.2 Mild replication stress reduces fork speed and causes DNA breaks. (A) Illustration of experimental design. PonA-treated H1299 E1 and D1 cells were cultured in the presence of hydroxyurea (50 uM) for 48 h, then sequentially pulsed with CldU and IdU. (B) DNA fiber analysis of fork velocity in H1299 E1 and D1 cells cultured as in (A). (C) Comet assay analysis of DNA breaks in H1299 E1 and D1 cells cultured as in (A).

3.3.2 Restoration of dTTP pools rescues replication defects in FHIT-deficient cells

To determine if dTTP pool imbalance caused the replication defects in FHIT-deficient HEK293 cells, FHIT-silenced cells were supplemented with thymidine for 48 h, providing fresh thymidine every 24 h. dNTP measurements confirmed that thymidine supplementation for 48 h resulted in an increase in dTTP pools in both control and FHIT-silenced HEK293 cells. Thus, supplementation is sufficient to restore dTTP pools in FHIT-deficient cells (Figure 3.3).
Figure 3.3  Thymidine supplementation restores dTTP pools in FHIT-silenced cells. dNTP pools in siRNA transfected HEK293 cells supplemented with thymidine 10 µM. Bar graphs illustrate the means of 1 experiment performed in quadruplicate. Error bars show the standard deviations. dT = thymidine.

DNA fiber analysis of replication fork dynamics revealed that thymidine supplementation fully rescued fork velocity in FHIT-deficient cells to a mean velocity similar to control, FHIT-expressing cells (Figure 3.4A). Thymidine supplementation also improved the symmetry of sister replication forks proceeding outward from a common origin (Figure 3.4B). To determine if rescue of fork progression in FHIT-deficient cells could also prevent DNA break formation, we supplemented cells with thymidine and measured neutral comet tail moments. This analysis revealed that thymidine supplementation fully prevented the DNA breaks caused by FHIT knockdown (Figure 3.4D). The results confirm that the supply of dTTP in the FHIT-deficient condition was inadequate to support efficient DNA replication and that the replication defects are the source of the spontaneous DNA breaks in FHIT-deficient cells.
Figure 3.4 Restoration of the dTTP pool rescues replication defects and prevents chromosome breakage in FHIT-deficient cells. (A) DNA fiber analysis of fork velocity in siRNA transfected HEK293 cells supplemented with 10 µM thymidine. Statistical significance was determined using a 2-sided Student’s T-test (siCtrl mock, n = 136; siCtrl + dT, n = 152; siFHIT mock, n = 155; siFHIT + dT, n = 153). (B) Sister fork asymmetry in siRNA transfected HEK293 cells supplemented with 10 µM thymidine, (siCtrl mock, n = 87; siCtrl + dT, n = 86; siFHIT mock, n = 96; siFHIT + dT, n = 93). ns = not significantly different. (C) Neutral comet assays of HEK293 cells with FHIT knockdown, untreated or supplemented with 10 µM thymidine, (siCtrl mock, n = 242; siCtrl + dT, n = 156; siFHIT mock, n = 193; siFHIT + dT, n = 115). Statistical significance was determined using the Kruskal-Wallis rank sum test.
3.3.3 FHIT activates S phase-specific expression of TK1 for thymidine salvage

dTTP is synthesized by two pathways, the *de novo* pathway via thymidylate synthase, TYMS; and the scavenger pathway via thymidine kinase 1, TK1 (Hu and Chang, 2007). Because of the observed effect on dTTP pools, we evaluated expression of TK1 and TYMS enzymes following FHIT knockdown. Expression of TYMS was unaffected by FHIT-silencing; in contrast, TK1 expression was severely depleted in HEK293 cells transfected with *FHIT* siRNAs (Figure 3.5A).

**Figure 3.5 FHIT is positively associated with TK1 expression.** (A-D) Western blot analysis of TK1 and TYMS expression in siRNA transfected HEK293 cells (A), in siRNA transfected HEK293 cells with or without exogenous FHIT overexpression (B), in *Fhit*+/+ and *Fhit*−/− mouse kidney epithelial cells (C), or in A549 cells with FHIT stably knocked down for 7-9 weeks (D).
Exogenous overexpression of FHIT in FHIT-deficient cells restored TK1 expression to normal levels (Figure 3.5B). *Fhit*−/− mouse kidney cells also expressed trace levels of TK1 compared to *Fhit*+/+ cells (Figure 3.5C), and stable FHIT knockdown in A549 lung cancer cells caused TK1 down-modulation for at least 9 weeks (Figure 3.5D). Thus, FHIT modulation of TK1 expression is a general phenomenon, necessary for producing sufficient dTTP for DNA synthesis.

The positive regulation of TK1 by FHIT may either occur through activation of mRNA transcription, protein translation or by restricting TK1 protein degradation to mitosis (Ito and Conrad, 1990; Ke and Chang, 2004; Stewart et al., 1987). As an initial step to determine at what level FHIT regulates TK1 expression, the proteasome inhibitor, MG132, was used to block TK1 degradation. In this experiment, steady-state levels of TK1 will increase with prolonged proteasome inhibition, with the rate of TK1 increase a function of the rate of protein synthesis. Intriguingly, MG132 treatment led to a very modest increase in TK1 expression in FHIT-deficient cells and a robust increase in expression in control cells, suggesting that FHIT stimulates protein synthesis (Figure 3.6A and B). Using the reverse strategy by blocking protein synthesis with cycloheximide, steady-state levels will decrease with prolonged inhibition, and the rate of TK1 decrease as a function of the rate of protein degradation. Using cycloheximide to block protein synthesis, we found that FHIT did not enhance TK1 protein stability as TK1 levels decreased at the same rate in control and FHIT-silenced cells (Figure 3.6C and D). Thus, FHIT stimulates TK1 protein production.
Figure 3.6  Fhit promotes TK1 protein up-regulation during S-phase. (A) Western blot of TK1 and GAPDH in siRNA transfected HEK293 cells treated for the indicated times with the proteasome inhibitor, MG132 (10 µM). (B) Relative TK1 protein expression determined by densitometric analysis of western blots from (A). (C) Western blot of TK1 and GAPDH in siRNA transfected HEK293 cells treated for the indicated times with the translation inhibitor, cycloheximide (CHX, 100 µg/ml). Protein lysates from siFhit cells were loaded in gels at a mass ratio 3:1 compared to siCtrl lysates in order to more accurately measure rate of degradation. (D) Relative TK1 protein expression determined by densitometric analysis of western blots from (C). (E) Western blot of TK1 and Cyclin A2 in siRNA transfected HEK293 cells synchronized by the thymidine/nocodazole block and released for up to 24 h. Cells pass through S and G2 phases between 16-24 h after release as determined by Cyclin A2 expression. The first lane for the siCtrl blot shows TK1 expression and Cyclin A2 expression in siFhit cells for a direct comparison of siFhit and siCtrl samples. This was also done for the siFhit blot on the right.
TK1 expression is regulated in a cell-cycle dependent manner at the transcriptional and the translational levels (Ito and Conrad, 1990). During G1, TK1 expression is minimal. TK1 expression is drastically increased during S phase and peaks during G2. Then as cells complete cell division, TK1 is rapidly degraded in a proteasome-dependent manner (Ke and Chang, 2004). Restriction of TK1 expression to the S and G2 phases of the cell cycle ensures that dTTP levels are tightly regulated and only synthesized when needed for DNA synthesis or repair (Hu and Chang, 2007). Because FHIT was shown to up-regulate TK1 expression, we asked whether this positive regulation was S phase specific. For this, we synchronized cells to mitosis using a thymidine/nocodazole synchronization protocol. Release from the nocodazole block led to a rapid degradation of TK1 levels in both FHIT-expressing and FHIT-deficient cells. Interestingly, as cells entered S phase, TK1 expression increased in control cells peaking during G2. In contrast, FHIT-deficient cells failed to up-regulate TK1 throughout S phase and only minimally expressed TK1 in G2 (Figure 3.6E).

3.3.4 FHIT protects TK1 mRNA from degradation

Because the S phase – specific synthesis of TK1 protein is regulated by the G1/S activation of TK1 mRNA transcription and the S phase activation of TK1 protein translation, FHIT may function to enhance either TK1 transcription or translation. We compared the TK1 mRNA levels in HEK293 cells and observed a 60-70% decrease in the relative expression of TK1 in FHIT-silenced cells (Figure 3.7A). Similar results were obtained in HCT116 following FHit knockdown (Figure 3.7B), suggesting that FHIT promotes S phase TK1 enzyme levels by controlling TK1 mRNA expression.
mRNAs are capped at their 5’ end. The 5’ cap serves to recruit translation factors that load the ribosome for cap-dependent translation and also acts to protect mRNA from 5’-3’ exonuclease-mediated degradation (Mukherjee et al., 2012). Interestingly, a substantial fraction of TK1 mRNA reportedly exists in a 5’-uncapped state and can be translated in a cap-independent manner (Chou and Chang, 2001; Mukherjee et al., 2012). This would suggest that uncapped TK1 mRNA is a translationally active entity, despite the mRNA degradation pathway that targets uncapped mRNA. Mechanisms that prevent degradation of uncapped TK1 mRNA would increase both mRNA and protein levels. To determine whether FHIT protects TK1 mRNA from degradation, we measured TK1 mRNA stability in control and FHIT-silenced HEK293 cells. Actinomycin D was used to block transcription and RNA was extracted 0, 2, 4 and 8 h after actinomycin D addition.

Figure 3.7  FHIT protects TK1 mRNA from degradation. (A and B) qRT-PCR analysis of TK1 mRNA levels in siRNA transfected HEK293 (A) or HCT116 (B) cells. TK1 mRNA expression were normalized to GAPDH mRNA levels. (C) qRT-PCR analysis of TK1 mRNA expression in siRNA transfected HEK293 treated with actinomycin D for 0-8 h. TK1 mRNA expression was normalized to GAPDH mRNA expression.
The rate of degradation was nearly identical for control and FHIT-deficient cells during the first 2 hours of the time-course; however, by 4 h TK1 mRNA levels stabilized to a resting state that was 3-fold higher in control cells compared to FHIT-silenced cells (Figure 3.7C). Thus, FHIT protects the basal TK1 mRNA levels from degradation.

Xrn1 is the main 5’-3’ exonuclease that binds and degrades uncapped mRNA (Braun et al., 2012). We tested whether FHIT protected TK1 mRNA from Xrn1-mediated degradation. To this end, HEK293 cells were transfected with siRNAs targeting FHIT, XRNI, or non-targeting control siRNAs and TK1 mRNA and protein levels were assessed. As expected, FHIT knockdown led to a 60% decrease in TK1 mRNA and a corresponding decrease in TK1 protein. However, Xrn1 silencing failed to rescue the decrease in TK1 mRNA and protein levels in FHIT-silenced cells (data not shown). Thus it would seem that the decrease in TK1 in FHIT-deficient cells is caused by Xrn1-independent degradation of TK1 mRNA.

Collectively, the results show that FHIT protects the integrity of the genome by preventing replication stress. FHIT supports dTTP production by enhancing the S phase-specific expression of TK1 mRNA and enzyme, and FHIT increases TK1 mRNA levels by protecting them from degradation. In short, FHIT stabilizes genomic DNA by stabilizing TK1 mRNA.

3.4 Discussion

3.4.1 Nucleotide salvage and chromosome instability

FHIT loss was shown to induce genome instability, indicating that FHIT has a genome caretaker function. It was further shown that the genome caretaker function of
FHIT was to prevent spontaneous replication stress. Here, I have shown that, mechanistically, the replication stress in FHIT-deficient cells was caused by a decrease in dTTP pools: silencing FHIT expression led to a moderate decrease in dTTP, a reduction sustained in stably FHIT-silenced cells; thymidine supplementation rescued the replication defects and suppressed DSBs in FHIT-silenced cells. FHIT was shown to up-regulate TK1 enzyme expression for the timely production of dTTP during S phase via the thymidine salvage pathway (Figure 3.8).

![Figure 3.8 Model of FHIT/TK1 pathway to promote efficient DNA replication](image)

Figure 3.8  Model of FHIT/TK1 pathway to promote efficient DNA replication

TK1-deficiency is the proposed mechanism for replication stress in FHIT-silenced cells. Interestingly, the Tk1 knockout mouse also exhibits evidence of replication stress and chromosome instability. Examination of B lymphocytes and erythrocytes from Tk1−/− mice revealed a dramatic decrease in dTTP levels, activation of the replication stress
checkpoint kinase Chk1, and phosphorylation of the DNA damage marker H2AX (Austin et al., 2012). In another study it was shown that Tk1−/− reticulocytes and normochromic erythrocytes had a 5- and 8-fold increase in spontaneous micronuclei formation, respectively (Dobrovolsky et al., 2005). These mice are also partially immune-deficient, as are the Fhit−/− mice (Zanesi et al., 2001). These findings lend further credence to the FHIT/TK1 model where loss of FHIT causes reduced TK1, insufficient dTTP synthesis, and subsequent replication stress and genome instability.

3.4.2 dNTP pools and genome instability

dNTP pool depletion affects DNA replication, causes genome instability, and is likely involved in oncogenic transformation. Whereas oncogenes cause a dramatic decrease in dNTP levels, FHIT loss causes only moderate dTTP reduction, adequate to affect DNA synthesis negatively without blocking cell cycle progression. It is also relevant that deficiency of BLM helicase, in the highly penetrant autosomal recessive cancer-susceptibility syndrome, Bloom syndrome, is associated with a strong cytidine deaminase defect, leading to pyrimidine pool imbalance. Specifically, this results in a 50% increase in the dCTP pool and only a 17% decrease in dTTP levels (Chabosseau et al., 2011). In BLM-deficient cells, thymidine supplementation leads to reduction of sister chromatid exchange frequency and is sufficient for full restoration of replication fork velocity. FHIT loss causes a more significant reduction of dTTP than in BLM-deficient cells, ranging from a 25-50% decrease. It may be puzzling that thymidine supplementation can restore the supply of dTTP in FHIT-deficient cells, since TK1, down-modulated by FHIT-deficiency, is needed to convert thymidine to dTTP. Clearly
TK1 is not completely absent in FHIT-silenced cells, since BrdU, CldU and IdU are also phosphorylated by TK1 prior to incorporation into newly synthesized DNA. It is possible that by compensating with an elevated continuous supply of thymidine for 48 h, even with low TK1 expression, FHIT-deficient cells accumulate a sufficient supply of dTTP to support DNA synthesis.

3.4.3 Regulation of TK1

TK1 is regulated in a cell cycle-dependent manner, with minimal expression during most of G1-phase; as cells prepare to start S-phase, TK1 expression is dramatically up-regulated to contribute to dTTP biosynthesis. Control of TK1 expression occurs at the transcriptional and the translation level. TK1 protein expression remains high throughout S- and G2-phases to facilitate sufficient dTTP production and as cells near the end of mitosis the enzyme is rapidly degraded to prevent overproduction of dTTP (Hu and Chang, 2007). Accordingly, positive regulation of TK1 by FHIT is also S- and G2-phase-specific and occurs at the mRNA level. Actinomycin D experiments suggest that FHIT protects a substantial fraction of TK1 mRNA from degradation, and apparently independent of Xrn1-mediated degradation of uncapped mRNA. Nevertheless, there are significant amounts of uncapped TK1 mRNA that are translationally active (Chou and Chang, 2001; Mukherjee et al., 2012). It will be important to determine if FHIT protects the uncapped fraction from degradation.

How might FHIT affect TK1 mRNA stability? Perhaps it is prudent to consider the activities of related proteins within the same HIT family. Known HIT family members have mRNA scavenger decapping activity (Salehi et al., 2002). An interesting
example is HIT-45, an enzyme unique to trypanosomes but with closest homology to FHIT proteins. HIT-45 has scavenger decapping activity for the unique 5’ mRNA cap of trypanosomes; the catalytic histidine triad core is essential for HIT-45 activity (Banerjee et al., 2009). It is tempting to speculate that in certain contexts FHIT may bind normal or perhaps aberrant 5’ cap structures and stabilize the full length RNA. Indeed, the 5’ cap structure resembles dinucleoside triphosphates (Figure 3.9), some of which are FHIT substrates; FHIT mutants with poor substrate binding lack the genome caretaker function (Saldivar et al., 2013). There is at least one report claiming that FHIT can hydrolyze cap-like structures (Bojarska et al., 1999), so alternatively, FHIT may catalyze removal of aberrant cap structures that target the mRNA for degradation. Whatever the mechanism of FHIT function to protect TK1 mRNA, it will be important to discover how many other mRNAs are regulated through stabilization by FHIT and by extension what other cellular processes FHIT may modulate.
3.4.4 The FHIT/TK1/cancer paradox

An interesting paradox in the FHIT/TK1 pathway is that TK1 is often overexpressed in cancer cells while FHIT is reduced or absent. In fact, data obtained from public databases revealed that in 265 cancer cell lines and 354 lung tumor samples FHIT expression and TK1 expression had a strong negative correlation (correlation coefficient $= -0.251$). One possible explanation is that the apparent FHIT/TK1 pathway is an artifact of in vitro cell culture and not the mechanism of the DNA caretaker function of FHIT in vivo. Another explanation is that FHIT loss in the preneoplastic cells results in only a transient reduction in TK1 expression and dTTP pools. The ensuing replication stress and
genome instability in proliferating cells creates an environment of increasing genetic variation and strong selective pressure conducive for clonal expansion of mutants with an acquired growth advantage. In particular, FHIT-deficient precancerous cells would be under selection for mutants that can increase the supply of dTTP. A mutation that allows FHIT-independent TK1 expression would be a beneficial mutation, as would any mutation that increases the supply of dTTP (e.g., a mutation that increased TYMS expression). Thus, evidence for selective pressure to specifically raise intracellular dTTP levels would be the acquired up-regulation of TK1 or TYMS. Importantly, FHIT expression was strongly inversely correlated to that of TYMS (correlation coefficient = -0.219) in the same panel of cancer cell lines and lung tumor tissues. Thus, the observed overexpression of TK1 in FHIT-negative cancer cells may actually be a vestige of cells that evolved through an intermediary premalignant stage of dTTP shortage.

Experimental evidence supporting this explanation comes from following Fhit and Tk1 expression in kidney epithelial cells established from Fhit+/+ and Fhit−/− mice. Fhit expression in the +/- cells gradually decreases as cells are subcultured several times over a period of several months, with a corresponding decrease in Tk1 expression as is expected since Fhit is needed to stabilize Tk1 mRNA. In contrast, in Fhit−/− cells there is minimal Tk1 expression initially, but gradually Tk1 increases over the months of subculturing (data not shown). Thus, a Tk1-overexpressing mutant cell with adequate dTTP pools clonally expands and becomes the dominant population among the sister Tk1- and dTTP-deficient cells.
3.4.5 Chromosome fragile sites and FHIT

The finding that FHIT loss-induced genomic alterations were a consequence of dTTP pool depletion provides an intriguing twist to the ongoing narrative of chromosome fragile sites: the fragile \textit{FHIT} gene product may protect fragile sites. For example, there is a class of chromosome fragile sites, the folate-sensitive fragile sites, that are unstable under conditions that cause thymidylate depletion, including culturing in medium deficient in thymidine or folate. Thymidine supplementation rescues the fragility of these sites (Sutherland, 1979). Furthermore, folate-deficiency causes chromosome instability in human and mouse blood cells (Blount and Ames, 1995; MacGregor et al., 1990). These findings independently establish that an insufficient supply of dTTP can cause chromosome instability at specific loci and that the salvage pathway, via TK1 activity, is a required source of dTTP to support DNA synthesis. Interestingly, folate is an important nutrient that serves as a cofactor for dTTP synthesis via activation of TYMS. Studies show that folate-deficiency correlates with several types of cancer, linking dTTP availability and tumorigenesis (Rampersaud et al., 2002). This is consistent with our findings that loss of FHIT decreases dTTP availability and promotes tumorigenesis. Because FHIT-deficient cells have increased breaks/metaphase chromosome, it will be interesting to determine whether these breaks occur at non-random chromosome loci. If so, this would be evidence for a novel class of chromosome fragile sites, the so-called FHIT-sensitive fragile sites that are induced, ironically, by loss of the fragile \textit{FHIT} gene product.
Chapter 4. A Unified Model for the Origin of Genome Instability in Sporadic Cancer

4.1 Introduction

Cancer is a genetic disorder characterized by the presence of thousands of mutations, small and large DNA deletions, amplifications, translocation and inversions, and in most cases an abnormal number of chromosomes (Stratton, 2013; Stratton et al., 2009). Because of the highly unstable genomes of cancer cells, tumors are made up of heterogenous subpopulations, both genetically and morphologically (Heng et al., 2009). The highly unstable nature of tumor cell genomes means that the use of targeted therapies to treat cancer will have limited effectiveness, as new resistant clones will emerge (Schmitt et al., 2012). It may also mean that many of our sophisticated preclinical models of cancers are a biased representation of tumor behavior in that they are “driver” mutation-centric, and fail to account for the complex genetic, epigenetic and environmental interactions that take place during the premalignant stages of tumorigenesis (Ashworth et al., 2011; Urbach et al., 2012). Therefore, gaining a more complete understanding of the origin of genome instability and heterogeneity, and importantly, accurately modeling this process in preclinical studies will be a major step towards devising better therapies.
Genome instability occurs gradually: early preneoplastic lesions exhibit instability initially at chromosome fragile sites, whereas cancer cells have global instability (Tsantoulis et al., 2008). However, appearances can be deceiving as these observations are influenced by the clonal and subclonal composition of tumors and the limitations of our most sensitive methods for detecting mutations. Several new studies have uncovered widespread somatic mosaicism in humans, indicating that normal somatic cells are more genetically heterogenous than previously expected (Jacobs et al., 2012; Laurie et al., 2012; Young et al., 2012). Does the presence of somatic mosaicism imply the existence of cell populations with a higher rate and frequency of mutations, and possibly exhibiting a degree of genome instability, or does it reflect the gradual accumulation of mutations as we age? These questions pertain to when and how genome instability originates. It may be that at its origin, genome instability exists at a low level in near normal somatic cells, generating random mutations in only a small fraction of cells, undetectable by current sequencing methods until a mutation drives clonal expansion.

Much research has centered on understanding the causes of genome instability; many known factors contribute to its spread in sporadic cancers, including replicative stress, oxidative damage, telomere attrition and DNA repair defects (Negrini et al., 2010). Here, I discuss the leading hypotheses for how instability initiates tumor formation, including the mutator phenotype and oncogene-induced DNA damage models. I argue that these may be different phenotypes of cancer predisposition stemming from a common defect. I discuss the unique features of the FHIT-loss model and provide the
rationale for how FHIT-loss could be one of the underlying catalysts of the early events during tumorigenesis, supporting a unified model for the origin of genomic instability.

4.2 The mutator phenotype hypothesis

Normal proliferating cells possess robust mechanisms to ensure genomic integrity with each cell division. These mechanisms include DNA replication factories that copy DNA with remarkable fidelity, DNA repair pathways to correct any replication mistakes or repair DNA damage caused by environmental and endogenous agents, and mitotic checkpoints that promote faithful segregation of sister chromatids to daughter cells. As a result, proliferating cells are able to limit the number of spontaneous mutations to less than 1 mutation per 10 billion nucleotides per cell division depending on the cell type (Nikolaev et al., 2012).

In contrast, cancer cells often have thousands of mutations, with different cells within a tumor harboring synonymous and unique mutations (Schmitt et al., 2012). In order to account for the large numbers of mutations in cancer cells, Lawrence Loeb first postulated that cancer cells express a “mutator phenotype”, that is they have an elevated mutation rate compared to that of nonmalignant cells (Loeb, 2011). The development of a mutator phenotype is hypothesized to be an early step in tumorigenesis, due to the necessity of multiple cooperating oncogenic mutations to initiate and sustain the neoplastic process (Loeb, 2010). An elevated mutation rate would generate mostly random, nearly neutral mutations; however, upon acquisition of an oncogenic, tumor initiating mutation, selective clonal expansion would ensue capturing previously occurring passenger mutations within the given cell lineage. Throughout tumorigenesis,
cancer-driving mutations occur at random and multiple rounds of clonal selection generate a tumor made up of a predominant clone and several subclones. Thus, the mutator phenotype is integral to the clonal evolution of cancer, facilitating the acquisition of the different cancer hallmarks (Yates and Campbell, 2012).

So how do normal or precancerous cells acquire a mutator phenotype? The model proposes that random genome-wide DNA damaging events, either from environmental or endogenous sources, produce random mutations. By chance, a mutation within a genome maintenance gene, such as a DNA repair gene, would destabilize the genomic integrity of the affected mutant cell (Figure 4.1) (Loeb, 2010).

![Figure 4.1 The mutator phenotype hypothesis.](image-url)

**Figure 4.1 The mutator phenotype hypothesis.** In the early stages of tumorigenesis, a mutation in a DNA caretaker gene elevates the mutation rate in all descendant cells generating a heterogenous population. An oncogenic mutation drives clonal expansion. This process yields tumor cells made up of tens of thousands of clonal, subclonal, and random mutations that may be selected for when the environment changes. The blue box represents the mutator mutation; the yellow and black boxes represent passenger mutations; and the red box represents a driver mutation. The green box represents a subclonal mutation that is selected for upon changes in the environment.
There are probably hundreds of the so called “DNA caretaker” genes, and any one of these genes could potentially be a mutator gene, so long as a mutation elevates the mutation rate without significantly reducing cellular fitness. Cancers that require multiple mutation “hits” are predicted to be more dependent on a mutator phenotype as the probability of acquiring the necessary cooperating mutations is significantly increased when the mutation rate is elevated (Loeb, 2011). In support of the mutator phenotype, many familial cancer syndromes are caused by inherited mutations in DNA caretaker genes.

It is undisputed that tumor cells of most cancers have accumulated thousands of mutations and even tens of thousands, but it has been argued that the increased proliferation rate and longevity of tumor cells is sufficient to account for the number of mutations present in cancer cells, without postulating a mutator phenotype (Bodmer et al., 2008). Indeed, the time between carcinogen exposure and clinical appearance of a tumor can take more than 20 years. Nevertheless, estimates of mutation rates in cells following the cancer-initiating event are 200-fold higher than the rate in normal cells, suggesting that in the early stages of tumorigenesis, cells express a mutator phenotype (Bielas et al., 2006; Nikolaev et al., 2012).

4.3 Oncogene-induced replication stress and DNA damage

The oncogene-induced DNA damage model proposes that for many sporadic cancers, driver mutations in oncogenes dysregulate S phase entry causing aberrant proliferation, replication stress and DNA breaks in preneoplastic lesions (Halazonetis et al., 2008). This leads to chromosome instability producing aberrations throughout the
genome. Replication stress and DNA damage have been reported in human lung hyperplasias, dysplastic nevi of the skin, primary urinary carcinomas, colon adenomas and carcinomas, glioblastomas, and other preneoplastic tissues, and the assumption has been that oncogene activation is the instigator. Indeed, experimentally induced oncogenes in mouse and human cell culture models cause replication stress and DNA damage (Bartkova et al., 2006; Di Micco et al., 2006).

Oncogenes that cause constitutive mitogenic signaling have been shown to induce this type of DNA damage, including Ras, Cyclin E, E2F1, and Mos. In addition viral oncogenes, such as E6 and E7 of the HPV virus, have been observed to induce replication stress and DNA damage (Bester et al., 2011). All of these oncogenes act at different steps in the mitogenic signaling pathways and yet produce a common end result: impairment of the Rb-controlled G1 restriction point followed by premature S phase entry and a deregulated origin firing program.

Premature S-phase entry and inappropriate origin firing are both believed to produce replication stress through partially overlapping mechanisms. Becker et al. (2011) demonstrated that premature S phase entry of primary keratinocytes, due to transduction with an oncogenic virus, results in an inadequate supply of DNA precursors needed to support efficient DNA replication, and thereby causes replication fork defects, DNA breaks and chromosomal alterations. In this article it was reported that the viral oncogenes induced expression of many S-phase-specific factors and DNA replication proteins, but failed to up-regulate the necessary nucleotide biosynthetic enzymes. Furthermore, by directly supplementing cells with the needed DNA precursors, the
authors were able to suppress oncogene-induced DNA damage and prevent cell transformation. In agreement with these findings, it was recently shown that the HRAS (G12V) oncogene also caused a dNTP shortage in normal human fibroblast cells. This shortage was caused by decreased expression of thymidylate synthase and ribonucleotide reductase, and resulted in oncogene-induced senescence (Mannava et al., 2013). Thus, aberrant proliferation caused by oncogene activation and attenuation of the G1 restriction point precludes sufficient preparation for the essential task of DNA duplication leaving cells susceptible to developing chromosome alterations.

Inappropriate origin firing leading to an increase in the density of active replication forks is also of source of replication stress as it increases the frequency of replication fork collision with transcription machinery (Bermejo et al., 2012). Interference between replication and transcription can also be caused by the formation of RNA/DNA hybrids associated with transcription that hinder DNA unwinding. RNA/DNA duplexes are more stable and have been shown to induce DNA breaks and recombination (Wahba et al., 2011). A genome-wide screen designed to identify genome maintenance genes unexpectedly revealed a widespread role of RNA processing factors to promote the stability of the genome. Interestingly, many of these RNA factors have been shown to minimize the formation of aberrant RNA/DNA structures (Paulsen et al., 2009). RNA/DNA hybrids can be particularly detrimental at genes highly expressed during S phase, like those encoding histone proteins, as the frequency of replication and transcription interference is predicted to be increased at such genes (Tuduri et al., 2009).
Oncogene activation has also been shown to disrupt the normal origin firing program and increase the density of active replication forks, and accumulating evidence suggests that such collisions underlie the replication stress and DNA damage caused by oncogenes (Jones et al., 2012). Jones et al. (2012) found that overexpression of the oncogene, Cyclin E, disrupts the normal origin firing program, increasing the density of active replication forks. As a consequence, interference between replication and transcription frequently led to reduced fork progression, increased DNA damage and recombination. Common fragile sites are particularly susceptible to oncogene-induced replication stress, and intriguingly many fragile sites overlap large genes (Helmrich et al., 2006). Large genes require a longer time to transcribe and have been shown to have elevated collision frequencies between replication forks and transcription machinery or RNA/DNA duplexes (Helmrich et al., 2011). These observations imply that interference between replication and transcription may underlie the fragility of some chromosome loci and provide further evidence that oncogene-induced DNA damage is a consequence of a dysregulated origin firing program.

It is likely that these two mechanisms of oncogene-induced DNA damage are overlapping. For example, evidence suggests that a decrease in dNTPs slows replication fork progression, and cells respond by firing back-up origins increasing the density of active replication forks (Gay et al., 2010; Ge et al., 2007). Thus, a shortage in dNTPs due to premature S-phase entry could indirectly increase the frequency of replication/transcription interference. Alternatively, increased origin firing and a subsequent increase in active replication forks will increase the demand for cellular
resources needed to support DNA replication, and without a corresponding increase in supply, there will likely be a shortage of DNA precursors (Figure 4.2) (Petermann et al., 2010b).

**Figure 4.2** The oncogene-induced DNA damage model of genome instability.

### 4.4 Unresolved problems regarding current models for the origin of genome instability

While both the mutator phenotype and oncogene-induced DNA damage models have many prominent supporters, it is curious that often support is given exclusively to one model or the other. This is because for both models many enigmatic observations in
experimental systems and in tumor samples have remained unexplained. However, it is my opinion that these discrepant views are resolvable with the emergence of the FHIT loss-induced model of genome instability. In the remaining sections of this chapter, I will elaborate on how FHIT function and expression resolves the main problems of each model, and conclude with a unified model explaining how instability initiates in cancer genomes.

4.4.1 *FHIT* as a mutator gene

One of the main criticisms of the mutator phenotype is the apparent lack of candidate mutator genes, (i.e. a DNA caretaker gene or genes that are frequently mutated in the early lesions and result in an elevated mutation rate). Many cancer genomes have now been sequenced and each year the number of sequenced cancer genomes steadily increases. These large genomic studies have revealed a puzzling trend: many cancer genomes have thousands even hundreds of thousands of mutations, yet there are relatively few mutations involving known DNA caretaker genes (Negrini et al., 2010). This has led some to argue that genomic instability in cancers is not caused by a mutator phenotype but rather by oncogene-induced DNA replication stress. However, with the finding that FHIT has a DNA caretaker function through support of DNA replication (Saldivar et al., 2012), the *FHIT* gene is predicted to be a candidate mutator gene, as *FHIT* deletions do occur in the early stages of cancer development and are among the most frequent mutations in cancer. FHIT loss specifically causes chromosome instability, yet cancers also exhibit thousands of base-substitution mutations and small insertions and
deletions. Thus, for *FHIT* to be a good candidate mutator gene, *FHIT* inactivation must also account for the presence of these point mutations and small insertions and deletions.

New evidence now suggests that replication stress may be a source of both point mutations and small insertions and deletions. Studies in yeast, mammalian cells and cancer cells have begun to reveal that chromosome alterations caused by replication stress can produce a mutator phenotype. Poli et al. (2012) investigated DNA replication in different mutant yeast strains under chronic replication stress. Interestingly, they found that yeast mutants that have spontaneous DNA breaks and a chromosome instability phenotype adjusted to chronic replication stress by increasing dNTP pools (Poli et al., 2012). Davidson et al. (2012) made a similar observation, but further showed that the elevated dNTP pools increased the mutation rate and conferred a mutator phenotype (Davidson et al., 2012). It is possible that a similar adaptation occurs in FHIT-deficient cells where there is an initial shortage of dTTP and replication stress, but then a later expansion of the pools leading to a mutator phenotype; however, this has yet to be determined. Separate studies in yeast have shown that break-induced replication at collapsed replication forks is error prone producing clusters of base-substitution mutations and small insertions and deletions (Iraqui et al., 2012). Repair of DSBs by non-homologous end joining can also produce small insertions and deletions (Lieber, 2010; Villarreal et al., 2012), and repair by homologous recombination repair of DSBs, though considered to be error free, has now been shown to introduce clustered point mutations and small insertions and deletions (Deem et al., 2011). There is also evidence for replication stress-induced mutations in cancer cells from several sequenced cancer
These studies have revealed the presence of non-random, clustered mutations in cancer genomes (Roberts et al., 2012). The pattern of local hypermutation and mutation clusters suggests that they arose simultaneously, and because they frequently map to chromosome breakpoints it is likely they were generated during repair of a DSB or a collapsed replication fork (Nik-Zainal et al., 2012). Finally, a recent study showed that premalignant colon adenomas exhibited a mutator phenotype, and the authors concluded that replication stress was a major contributor to the elevated mutation frequency (Nikolaev et al., 2012). The collective evidence suggests that replication stress can produce a mutator phenotype, making the *FHIT* gene a strong candidate mutator gene (Figure 4.3).

**Figure 4.3 FHIT as a candidate mutator gene.** *FHIT* gene alterations occur in normal and precancerous cells due to environmental and endogenous sources of DNA damage. Upon loss of FHIT protein expression, cells accumulate chromosome alterations. Replication stress produces a mutator phenotype characterized by small insertions and deletions and base substitutions. This process facilitates the acquisition of oncogenic mutations followed by selective clonal expansion.
In the past year few years, published studies have begun to reveal the mutational signatures of cancer genomes (Alexandrov et al., 2013). From these mutation signatures, the mechanistic processes that generate the mutations can be inferred. To date, the mutation signatures in breast cancer and lung cancer have been the most extensively analyzed. If FHIT loss triggers a mutator phenotype, then the mutation signatures of breast and lung cancer should reflect the defective processes in FHIT-deficient cells, as FHIT loss is an early event in both of these types of cancer. Some of the signatures reveal unique mutational processes specific to a particular type of cancer. For example, lung cancers exhibit a signature of carcinogen-induced mutations, such as G > T transversions. G > T mutations are caused by polycyclic aromatic hydrocarbons present in cigarette smoke (Pleasance et al., 2010). Some signatures are common to several types of cancer, such as TpC > G and TpC > T mutations, present in breast, cervical and lung cancer (Nik-Zainal et al., 2012; Pleasance et al., 2010; Stephens et al., 2012). These mutations are caused by deamination of methylated cytosines by the APOBEC family of enzymes (Burns et al., 2013); however, it is presently unknown what causes elevated APOBEC-induced mutations in cancer cells. Interestingly, APOBECs preferential target methylated cytosines present in ssDNA, and long stretches of ssDNA accumulate at stalled replication forks and DNA breaks. Accordingly, the increased APOBEC-induced mutations in cancer cells are the result of increased replication stress and chromosome breaks (Roberts et al., 2012). As replication stress and chromosome breaks are induced by FHIT-deficiency, the APOBEC mutation signature may be a mutator phenotype expressed in FHIT-deficient cells.
The final two mutation signatures I discuss here are T > G transversions and T > C transitions present in BRCA1- and BRCA2-deficient breast cancers (Nik-Zainal et al., 2012). T > C transitions are also a mutation signature in lung cancer (Pleasance et al., 2010). The process inducing these mutations is unknown, but they may be a specific signature of a defect in FHIT-deficient cells. Because FHIT-deficient cells have decreased dTTP pools, it is assumed that the ratio of dUTP/dTTP is significantly increased and the rate of dUTP misincorporation over dTTP is concomitantly increased. Notably, dUTP incorporation into genomic DNA is a major source of abasic sites due to enzymatic removal of uracil by uracil DNA glycosylase (Guillet and Boiteux, 2003), and alkaline comet assay results suggest that FHIT-deficient cells have increased abasic sites (data not shown) supporting the likelihood of increased dUTP incorporation in place of dTTP. Depending on the translesion polymerase, DNA replication preferentially inserts a guanine or a cytosine across from abasic sites (Prakash et al., 2005; Waters et al., 2009), and following another round of DNA replication results in a T > C or a T > G mutation. Accordingly, studies in yeast have shown that mutants with increased dUTP incorporation have a 100- to 300-fold increase in T > C and T > G mutations, respectively (Collura et al., 2012). It is interesting that both T > C and T > G mutations are common in BRCA1- and BRCA2-deficient breast cancer and T > C mutation are common in lung cancer, as loss of FHIT expression occurs very frequently in these types of cancer. Thus, it is possible that the T > C transition and the T > G transversion may be specific mutation signatures of FHIT-deficient cells (Figure 4.4 and 4.5).
Figure 4.4 Mutation signatures in lung cancer. In the box on the left are the 4 mutation signatures associated with lung cancer. Carcinogen-induced mutations due to cigarette smoke produces C > A mutations. These carcinogens also silence FHIT expression. APOBEC enzymes induce a second class of mutations in lung cancer. APOBECs preferential mutate methylated cytosines in ssDNA at stalled and collapsed forks. They also produce clusters of mutations at DNA breaks and at chromosome break points. Because FHIT loss promotes fork stalling, collapse, and chromosome breaks, FHIT loss may explain the high-prevalence of APOBEC-induced mutations in lung cancer. T > C mutations are a 3rd signature of mutations in lung cancer; their origin is unknown, but may be due to decreased dTTP pools in FHIT-deficient cells.
Figure 4.5 The T > C mutation signature of lung cancer may be due to decreased dTTP pools in FHIT-deficient cells. (A) Decreased dTTP pools in FHIT-deficient cells would increase the dUTP/dTTP ratio. (B) Increased dUTP/dTTP ratio increases the rate of dUTP misincorporation (step 2). Uracil DNA glycosylase removes uracil from DNA leaving an abasic nucleotide. Translesion polymerases frequently insert guanines and cytosines opposite abasic sites (step 3), resulting in T > G and T > C mutations (step 4).

The presence of mutation clusters associated with repair of DSBs or collapsed forks in cancer genomes and the studies in yeast showing that replication stress increases dNTPs and causes a mutator phenotype suggest that FHIT loss-induced replication stress and DNA breaks could produce a mutator phenotype in precancerous cells. Furthermore, the deciphering of mutational signatures in cancers that match the predicted mutational signature of FHIT-deficient cells suggests that FHIT-loss may initiate a mutator phenotype. However, experimental evidence is needed to determine if FHIT is a mutator
gene. To obtain in vivo evidence that FHIT-deficient cells express a mutator phenotype, Miuma et al. (manuscript in review) performed whole exome sequencing analysis on liver tissue DNA from Fhit+/+ and Fhit−/− mice. Intriguingly, Fhit−/− liver tissue had an approximate 2-fold increase in the number of somatic small insertions and deletions, and an estimated 3-fold increase in base substitutions. It is important to note that these mutations were present in untreated, normal liver tissue. It is unknown whether the increased mutations in the Fhit−/− tissues are due to replication defects and an increased dUTP/dTTP ratio. Further analysis of this data set will hopefully illuminate the source of increased mutations in Fhit-deficient cells. Nevertheless, these findings support the prediction that the FHIT gene, when inactivated, results in a mutator phenotype.

4.4.2 FHIT loss as a mechanism for evasion of oncogene-induced senescence

As stated previously, because of the apparent lack of mutator genes in cancer, oncogene-activation has been proposed to be the main source of genome instability in cancer cells. Despite the evidence in support of the oncogene-induced DNA replication stress and damage model, there is considerable opposition to its purported role in initiating genomic instability. It has been argued that because many chromosome-stable cancer cells similarly express the same oncogenes that are proposed to induce instability, it is not actually oncogene activation that causes instability (Cahill et al., 1999). This argument has recently been supported by the finding that chromosome instability in colorectal cancer cells is caused by loss of suppressor genes on chromosome arm 18q and not by oncogene activation (Burrell et al., 2013). Furthermore, nucleoside supplementation rescued the replication stress and chromosome instability in the 18q-
deficient cells, suggesting that the loss of suppressor genes caused the decrease in dNTP precursors rather than oncogene activation. This is consistent with the finding that loss of FHIT in cells of other organs causes replication stress and chromosome instability.

A second argument is based on the observation in normal and preneoplastic cells that oncogene-induced DNA damage results in cellular senescence and apoptosis, mediated by DNA damage checkpoint pathways. DNA damage checkpoints form a tumorigenesis barrier preventing the development of cancerous lesions from precancerous cells (Bartkova et al., 2005). p53 and ATM are central to the senescence barrier to tumorigenesis, and without mutational inactivation or experimental manipulation of these or other key components within the checkpoint pathway, oncogenes fail to transform cells (Lowe et al., 2004). Oncogene activation also induces senescence in a DNA damage-independent way through the Arf-p53 pathway (Courtois-Cox et al., 2008). It is unlikely that genomic instability would develop in senescent cells, yet it is still commonly suggested that oncogene-induced genomic alterations facilitate mutational inactivation of checkpoint proteins (Halazonetis et al., 2008). This is also unlikely as evidence indicates that senescence is irreversible.

Despite the tumorigenesis barrier that senescence poses in response to oncogene activation, tumorigenic cells do acquire resistance to senescence. In fact, it is the senescence barrier that is believed to be a major selective force for the highly frequent mutations in TP53 and ATM found in cancer cells (Gorgoulis et al., 2005). Indeed, the oncogenes that cause DNA damage and senescence also transform cells in vitro and promote tumorigenesis in mouse models. So how do we reconcile these apparent
contradictions? An important point to make here is that in the in vitro models, oncogenes are activated in a very high number of cells and yet transformation occurs in fewer than 1 in $10^5$ cells. Furthermore, transgenic oncogenes that are ectopically expressed in mice induce clonal rather than systemic tumors (Nicholson and Duesberg, 2009). This is in contrast to human cancers, where oncogenes are not ectopically activated but become activated only in tumor cells. These observations confirm that driver mutations only work in the right genetic and environmental context or “soil” to support clonal expansion, underlying the importance of having a degree of genome instability and heterogeneity prior to oncogene activation (Nicholson and Duesberg, 2009). This is also in accord with the hypothesis that senescence is an irreversible process, and that pre-existing, non-senescent cells are selected for clonal expansion (Sarkisian et al., 2007).

How much heterogeneity is present in cells prior to oncogene activation is unknown, but recent findings conclude that heterogeneity is widespread in normal cells, increases with age and is a strong predictor of disease (Jacobs et al., 2012; Laurie et al., 2012; Young et al., 2012). Thus, it is possible that mutations that impair the DNA damage checkpoints may be present in some cells prior to oncogene-induced senescence. Such mutations only have a selective advantage in cells accumulating DNA damage, and thus would not be detectable in the early preneoplastic stages of tumorigenesis until after senescence evasion and clonal expansion. Evidence for this comes from follow-up studies of the DNA caretaker function of FHIT. In primary cells established from $Fhit^{+/+}$ and $Fhit^{-/-}$ mice we have discovered that the genome instability caused by Fhit loss leads to inactivation of the p53 pathway allowing evasion of senescence and resistance to
genotoxic agents (Miuma et al., manuscript in preparation). In mouse embryo fibroblasts, $Fhit^{-/-}$ cells incurred a chromosome alteration resulting in amplification of the $Mdm2$ gene. This amplification was selected for as cells underwent senescence and resulted in a rapid immortalization of $Fhit^{-/-}$ MEFs. In a separate model system, mouse kidney epithelial cells established from $Fhit^{+/+}$ and $Fhit^{-/-}$ mice were subcultured for several weeks or treated with a genotoxic drug to induce apoptosis. The $Fhit^{-/-}$ cells were more resistant to genotoxin treatment and resistant clones contained a $Trp53$ mutation within the DNA-binding domain leading to complete p21 silencing. The importance of these findings is that FHIT loss-induced genome instability produces spontaneous mutations which randomly affect the p53 pathway, and under selective pressure, p53 checkpoint-resistant clones emerge.

These findings do not diminish the importance of oncogenes, as oncogenes drive tumorigenesis. Instead, our work resolves the problem of oncogene-induced senescence. Oncogenes require inactivation of p53 pathway to transform. They contribute to the selective force for $TP53$ mutations, but evidence is lacking that they can induce $TP53$ mutations. $Fhit$-loss in mouse cells does cause p53 inactivation as a result of genome instability. Thus, oncogene activation and FHIT loss likely work in concert, with loss of FHIT inducing mutations, and oncogenes inducing the senescence barrier that selects for p53 inactivation (Figure 4.6).
4.5 Conclusions

In this final chapter, I have considered the DNA caretaker role of FHIT in the context of genome instability in sporadic cancer. I have attempted to merge the two predominant hypotheses for how instability initiates in precancerous cells, the mutator phenotype and oncogene-induced DNA damage models, and have argued that FHIT loss resolves some of the main objections to these two models. I have presented evidence that replication stress can produce a mutator phenotype and that the mutation signatures of
cancers that are predominantly FHIT-deficient match the predicted mutation signatures of FHIT loss-induced genome instability. Furthermore, the dTTP shortage in FHIT-deficient cells provides a mechanistic explanation for T > C and T > G mutation signatures in breast and lung cancer. I presented experimental evidence that p53 inactivation can occur through FHIT loss-induced mutations, providing an explanation for how cells overcome oncogene-induced senescence. In accordance with the fact that FHIT is an early target in precancerous cells, I propose a unified model for how genome instability frequently initiates in cancer: the FHIT gene is a candidate mutator. FHIT inactivation in carcinogen-exposed cells or in early preneoplastic cells triggers the expression of a mutator phenotype. Over the course of perhaps many years, genome instability generates many mutations and facilitates acquisition of both oncogenic-driver mutations and suppressor-inactivating mutations, and such cooperating mutations yield a genetic environment conducive for clonal expansion (Figure 4.7).
Loss of FHIT expression due to FRA3B alterations or promoter methylation is an early event in tumorigenesis, generating a cell lineage expressing a mutator phenotype. Driver mutations in oncogenes drive clonal expansion, but also senescence. Under this selective environment, mutations impairing the p53 pathway are selected for clonal expansion.
REFERENCES


APPENDIX A: Identified Copy Number Aberrations in Mice Tissues and Cell Lines

Table A.1 Complete list of copy number aberrations in \textit{Fhit}^{+/+} and \textit{Fhit}^{--} MEFs.

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<td>13A1</td>
<td>loss</td>
<td>1</td>
<td>Gm7446</td>
<td>13974389-13987592</td>
<td>13204</td>
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<tr>
<td>/-/- mouse 4 passage 3</td>
<td>16B3</td>
<td>loss</td>
<td>4</td>
<td>2010005H15Rik, Stfa1, Gm4758, BC117090</td>
<td>36257364-36321838</td>
<td>64475</td>
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<tr>
<td>/-/- mouse 4 passage 25</td>
<td>3A3</td>
<td>loss</td>
<td>1</td>
<td>Nlg1</td>
<td>25426121-25445541</td>
<td>19421</td>
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(Continued)
<table>
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<tr>
<th>Type</th>
<th>Passage</th>
<th>Loss</th>
<th>Genes</th>
<th>Chromosome</th>
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<tr>
<td>mouse 4</td>
<td>4E1</td>
<td>loss</td>
<td>2 Gm13109, Gm13101</td>
<td>143540903-143555553</td>
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<tr>
<td>mouse 4</td>
<td>13A1</td>
<td>loss</td>
<td>3 Ero1lb, LOC100502964, Gpr137b-ps</td>
<td>12696661-12720056</td>
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<tr>
<td>mouse 4</td>
<td>13A1</td>
<td>loss</td>
<td>1 Gm7446</td>
<td>13972917-13987592</td>
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<tr>
<td>mouse 4</td>
<td>16B3</td>
<td>loss</td>
<td>4 2010005H15Rik, Stfa1, Gm4758, BC117090</td>
<td>36257364-36321838</td>
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<tr>
<td>mouse 5</td>
<td>3A3</td>
<td>loss</td>
<td>1 Nlgn1</td>
<td>25426121-25471172</td>
</tr>
<tr>
<td>mouse 5</td>
<td>3F2.3-3F3</td>
<td>loss</td>
<td>4 Chia, Chi3l3, Chi3l4, Gm6522</td>
<td>105933348-106110585</td>
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<tr>
<td>mouse 5</td>
<td>4E1</td>
<td>loss</td>
<td>3 LOC100044633, Gm13083, Gm13088</td>
<td>143201660-143250513</td>
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<tr>
<td>mouse 5</td>
<td>4E1</td>
<td>loss</td>
<td>2 Gm13109, Gm13101</td>
<td>143540903-143555553</td>
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(Continued)
<table>
<thead>
<tr>
<th>Sample</th>
<th>Source</th>
<th>Chromosome</th>
<th>Gene(s)</th>
<th>Length</th>
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<tbody>
<tr>
<td>+/- mouse 5 passage 3</td>
<td>+/- mouse 5 passage 3</td>
<td>8C1</td>
<td>loss</td>
<td>1</td>
</tr>
<tr>
<td>+/- mouse 5 passage 3</td>
<td>+/- mouse 5 passage 3</td>
<td>13A1</td>
<td>loss</td>
<td>1</td>
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<td>+/- mouse 5 passage 3</td>
<td>+/- mouse 5 passage 3</td>
<td>16B3</td>
<td>loss</td>
<td>4</td>
</tr>
<tr>
<td>+/- mouse 5 passage 3</td>
<td>+/- mouse 5 passage 3</td>
<td>3A3</td>
<td>loss</td>
<td>1</td>
</tr>
<tr>
<td>+/- mouse 5 passage 25</td>
<td>+/- mouse 5 passage 25</td>
<td>3F2.3-3F3</td>
<td>loss</td>
<td>4</td>
</tr>
<tr>
<td>+/- mouse 5 passage 25</td>
<td>+/- mouse 5 passage 25</td>
<td>4E1</td>
<td>loss</td>
<td>2</td>
</tr>
<tr>
<td>+/- mouse 5 passage 25</td>
<td>+/- mouse 5 passage 25</td>
<td>8C1</td>
<td>loss</td>
<td>1</td>
</tr>
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</table>

(Continued)
| /-/- mouse 5 passage 25 | 10D2 | gain | 40 | Best3, Gm10747, Lrrc10, Cct2, Frs2, Yeats4, 9530003J23Rik, Lyz2, Lyz1, Cpfis6, Gm9002, Kifc5c, Gm9004, Cpm, Mdm2, S1c35e3, Nup107, LOC100504499, Rap1b, Mdm1, Il22, Gm9585, Gm9029, Gm9030, Gm9035, Ifitfb, Gm9044, I1ng, Dyrk2, LOC100417929, Cand1, LOC100418236, Grip1 | 116433036-119040590 | 2607555 |
| /-/- mouse 5 passage 25 | 13A1 | loss | 3 | Ero1lb, LOC100502964, Gpr137b-ps | 12699208-12720056 | 20849 |
| /-/- mouse 5 passage 25 | 13A1 | loss | 1 | Gm7446 | 13972917-13987563 | 14647 |
| /-/- mouse 5 passage 25 | 16B3 | loss | 4 | 2010005H15Rik, Stfa1, Gm4758, BC117090 | 36257364-36321838 | 64475 |
Table A.2  Copy number aberrations occur predominantly at fragile loci.

<table>
<thead>
<tr>
<th>CNA locus</th>
<th>MEF fragile site</th>
<th>Mouse lymphocyte fragile site</th>
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<tbody>
<tr>
<td>3A3</td>
<td>yes</td>
<td>Yes (medium expression)</td>
</tr>
<tr>
<td>3F2.3-3F3</td>
<td>yes</td>
<td>Yes (medium expression)</td>
</tr>
<tr>
<td>4E1</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>8C1</td>
<td>yes</td>
<td>Yes (high expression)</td>
</tr>
<tr>
<td>8C2-8C3</td>
<td>yes</td>
<td>Yes (high expression)</td>
</tr>
<tr>
<td>10D2</td>
<td>yes</td>
<td>Yes (medium expression)</td>
</tr>
<tr>
<td>13A1</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>16B3</td>
<td>no</td>
<td>Yes (medium expression)</td>
</tr>
</tbody>
</table>
Table A.3  Copy number aberrations in *Fhit*<sup>−/−</sup> tail tissue.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Chromosome</th>
<th>Status</th>
<th># of genes</th>
<th>Genes</th>
<th>Span</th>
<th>Size</th>
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</thead>
<tbody>
<tr>
<td>-/- tail</td>
<td>2D</td>
<td>loss</td>
<td>2</td>
<td>Olfr141, Olfr1094</td>
<td>86646195-86670052</td>
<td>23858</td>
</tr>
<tr>
<td>-/- tail</td>
<td>3F2.3-3F3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>loss</td>
<td>4</td>
<td>Chia, Chi3I3, Chi3I4, Gm6522</td>
<td>105933348-106110585</td>
<td>177238</td>
</tr>
<tr>
<td>-/- tail</td>
<td>4A4-4A5</td>
<td>loss</td>
<td>0</td>
<td>N/A</td>
<td>27102368-27307050</td>
<td>204683</td>
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<tr>
<td>-/- tail</td>
<td>4A4</td>
<td>loss</td>
<td>0</td>
<td>N/A</td>
<td>29717750-29825488</td>
<td>107739</td>
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<tr>
<td>-/- tail</td>
<td>6C2</td>
<td>loss</td>
<td>0</td>
<td>N/A</td>
<td>75342461-75464922</td>
<td>122462</td>
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<tr>
<td>-/- tail</td>
<td>8A4</td>
<td>loss</td>
<td>1</td>
<td>Sgcz</td>
<td>38773861-38831368</td>
<td>57508</td>
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<tr>
<td>-/- tail</td>
<td>8B1.3</td>
<td>loss</td>
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<td>N/A</td>
<td>52224120-52273067</td>
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<td>-/- tail</td>
<td>8B1.3</td>
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<td>52879756-52943783</td>
<td>64028</td>
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<td>-/- tail</td>
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<td>loss</td>
<td>2</td>
<td>Gm9892, Gm6463</td>
<td>53127775-53364507</td>
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<tr>
<td>-/- tail</td>
<td>8B1.3-8B2</td>
<td>loss</td>
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<td>N/A</td>
<td>55941523-56033681</td>
<td>92159</td>
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<tr>
<td>-/- tail</td>
<td>8C1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>loss</td>
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<td>Ttc29</td>
<td>80806273-80821978</td>
<td>15706</td>
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<td>N/A</td>
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<td>103011</td>
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<tr>
<td>-/- tail</td>
<td>13A1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>loss</td>
<td>1</td>
<td>Gm7446</td>
<td>13972917-13987592</td>
<td>14676</td>
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<tr>
<td>-/- tail</td>
<td>15B3.3-15C</td>
<td>loss</td>
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<td>N/A</td>
<td>49746931-49777457</td>
<td>30527</td>
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<tr>
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<td>N/A</td>
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<tr>
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<td>loss</td>
<td>4</td>
<td>2010005H15Rik, Gm13040, Gm13057, BC080695</td>
<td>36257364-36321838</td>
<td>64475</td>
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<td>60730307-60831232</td>
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</tbody>
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<sup>a</sup>CNAs also observed in *Fhit*<sup>−/−</sup> MEFs.