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Hits, Fhits and Nits: Beyond enzymatic function

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Introduction

Mammalian members of the Hit, Fhit and Nit protein families are predicted to have enzymatic functions due to their homology to other family members. Some of the enzymatic functions have been demonstrated *in vitro* and/or *in vivo*.

Hint and Fhit proteins are members of the Histidine Triad gene/gene product family with HxHxHxx motifs (where x is hydrophobic), a motif that is instrumental in the binding and cleavage of known substrates (Brenner, 2002). Before discovery of Fhit proteins as a branch of the HIT family, Hit proteins were mainly studied in bacteria and yeast (Huang *et al.*, 1995), but even in these organisms, the purpose of the enzymatic function was not known, though there have been many studies concerning the substrates (Ingram & Barnes, 2000).

The nitrilase superfamily consists of thiol enzymes involved in natural product biosynthesis and post-translational modification in plants, animals, fungi and prokaryotes. On the basis of sequence similarity and the presence of additional domains, the superfamily has been classified into 13 branches, nine of which have known or deduced specificity for specific nitrile- or amide-hydrolysis or amide-condensation reactions (Pace & Brenner, 2001). Genetic and biochemical analysis of the family member protein domains can help to predict subcellular localization, specificity and cell biology of uncharacterized protein sequences. Nitrilases were mainly known and studied in plants before discovery of the mammalian family members (Pace and Brenner, 2001) but substrates were not known for mammalian Nit family members until quite recently (Krasnikov *et al.*, 2009a,b). A multiple sequence alignment of amino acid sequences of Hint, Fhit and Nit family members is shown in Fig. 1, to illustrate the relationships among individual family members discussed here.

Hit Proteins

Hit enzymes are classified into three branches, with branches 1 and 2, the Hint and Fhit branches, exhibiting hydrolase activity (Brenner, 2002). The best characterized branch 1 enzymes are rabbit Hint and yeast Hnt1, which hydrolyze the adenosine 5'-monophosphoramidate substrate, AMP-lysine (Brenner *et al.* 1999).

Crystal structures of nucleotide-bound forms of Hint1 and Fhit demonstrated that the conserved residues in Hit proteins are responsible for their distinctive, dimeric structures with two identical purine nucleotide-binding sites (Lima *et al.*, 1996; Pace *et al.*, 1998).

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Hint-related proteins are found in all life forms, and Fhit-related proteins are found in animals and fungi. Hint homologs are intracellular receptors for purine mononucleotides whose cellular function remains unknown. Hints bind 2 purine nucleoside monophosphates per dimer and hydrolyze compounds such as adenosine monophosphoramidate (AMP-NH₂) and adenosine monophosphate (AMP)-lysine, with activity dependent on the middle Histidine (cited in Martin *et al.*, 2006). Activity on model compounds containing adenosine monophosphoramidate linkages provided evidence that Hint hydrolases reverse protein nucleotidylation. AMP-lysine hydrolase activities have been shown for Hint orthologs from yeast to humans (cited in Martin *et al.*, 2006). Lower specific activity AMP-lysine hydrolase activity is exhibited by Aprataxin, a Fhit domain containing enzyme inactivated in ataxia oculomotor apraxia 1 (AOA1, see below). In addition to Hint1, there are additional loci (Hint2 and Hint3) that encode Hint genes in humans (Brenner, 2002).

Yeast and mammalian Fhit homologs bind and cleave diadenosine polyphosphates (Ap(n)A) such as ApppA and AppppA (Huang *et al.*, 1995; Barnes *et al.*, 1996). Fhit-Ap(n)A complexes appear to function in a proapoptotic tumor suppression pathway in cancer-derived epithelial cells (Siprashvili *et al.*, 1997; Trapasso *et al.*, 2003). But for all these proteins, more is known about biological functions of the protein or protein-substrate complex than about the significance of the substrates and enzymatic catalytic products. In invertebrates, Fhit homologs are encoded as fusion proteins with polypeptide domains at the N-terminus that resemble plant and bacterial nitrilases, that may be candidate signaling partners in mammalian Fhit signal pathways (Pekarsky *et al.*, 1998; Pace *et al.*, 2000).

Hint1

Because of its structural similarity to Fhit, a tumor suppressor, Li *et al.*, (2005) used Hint1 knockout mice to study its role in tumorigenesis. At 2–3 years of age the spontaneous tumor incidence in Hint1^{-/-} mice was greater than in wild-type mice. Using a mouse model of carcinogen-induced mammary tumorigenesis, they reported a marked increase in the incidence of mammary and ovarian tumors in Hint1^{-/-} vs ^{+/+} mice. Hint1^{-/-} and ^{+/-} mice had similar tumor incidences, again similar to results with Fhit^{-/-} and ^{+/-} mice (Fong *et al.*, 2000; Zanesi *et al.*, 2001). Since the Hint1^{+/-} tumors retained expression of the unmutated wild-type allele, Hint1 also appears to be haplo-insufficient for tumor suppression. Hint1 has hydrolase activity toward phosphoramidate and acyladenylate (Bieganski *et al.*, 2002; Chou *et al.*, 2007), but these activities are not known to be relevant to its tumor suppressor pathway.

The underlying molecular mechanisms for Hint1 tumor suppressor function were examined and investigators reported that Hint1 participates in ionizing radiation (IR)-induced DNA damage responses (Li *et al.*, 2008). In response to IR, Hint1 is recruited to IR-induced foci and associates with γ H2AX and ATM. Hint1 deficiency impairs removal of such foci after DNA damage and the impairment is associated with impaired acetylation of γ H2AX and ATM and with retarded DNA repair after IR. Hint1 deficient cells are resistant to IR-induced apoptosis, suggesting that Hint1 tumor suppression is a result of its role in enhancing cellular responses to DNA damage.

Another study by the Weinstein group found that Hint1 up-regulates cellular p27 levels by: 1) inhibiting ubiquitylation by targeting the SCF(SKP2) ubiquitin ligase complex, and 2) inhibiting phosphorylation of p27 by Src *via* inhibiting Src expression (Cen *et al.*, 2009).

Aprataxin

Ataxia-ocular apraxia 1 (MIM 208920) is a frequent cause of autosomal recessive ataxia and shares neurological features with ataxia-telangiectasia, including early onset ataxia,

oculomotor apraxia and cerebellar atrophy, but does not share its extraneurological features (immune deficiency, chromosomal instability and hypersensitivity to X-rays). The gene causing AOA1 was discovered by two groups (Moreira *et al.*, 2001; Date *et al.*, 2001) to be a ubiquitously expressed protein designated Aprataxin (Moreira *et al.*, 2001). Authors noted that Aprataxin is a nuclear protein with a role in DNA repair somewhat similar to the function of the protein defective in ataxia-telangiectasia, but that would cause a phenotype restricted to neurological signs.

Aprataxin forms a distinct branch of the Hit domain superfamily of nucleotide hydrolases/transferases (Kijas *et al.*, 2006), has homology to polynucleotide kinase and encodes a forkhead-associated domain that facilitates interaction with XRCC1 (Reynolds *et al.*, 2009a,b). It can be sequestered in double-strand break repair foci and likely plays a role in single and double-strand break repair (Reynolds *et al.*, 2009a,b; Kijas *et al.*, 2006). Aprataxin also binds DNA and RNA, in support of the role of Aprataxin in the cellular response to DNA damage (Kijas *et al.*, 2006).

Most of the mutations identified in AOA1 are located within the Hit domain, consistent with a critical requirement for the Hit domain for normal neurological function. Many of these mutations reduce the stability and/or cellular level of Aprataxin and may thus be functionally null alleles. Aprataxin can remove AMP from the 5'-terminus of DNA breaks at abortive DNA ligation events, effectively 'proofreading' the DNA ligase reaction. In addition to the Hit domain, the C-terminal zinc finger is important for Aprataxin activity on 5'-AMP, probably to increase the affinity and/or specificity of Aprataxin for 5'-AMP substrates; it has not been determined if processing 5'-AMP termini arises *in vivo* (cited in Reynolds *et al.*, 2009a,b).

Hint2

Martin *et al.* (2006) showed that Hint2 is a mammalian-specific, nuclear-encoded mitochondrial Hint hydrolase that is predominantly expressed in the liver, is localized to mitochondria, sensitizes cells to apoptosis and is down-regulated in hepatocellular carcinoma. Protein adenylation, the covalent link of the AMP moiety of adenosine triphosphate to an amino acid residue of a protein occurs in bacteria, and several proteins in the liver have been reported to be adenylylated (cited in Martin *et al.*, 2006). It is not known if mitochondrial AMP adducts are the endogenous substrates of Hint2. Another possible substrate is adenosine monophosphoramidate (AMP-NH₂), found in eukaryotic cells but the biological significance of AMP-NH₂ and, in particular, in respect to apoptosis is unknown (cited in Martin *et al.*, 2006).

Fhit

Hydrolase activity

Fhit (EC 3.6.1.29), a member of the Hit nucleotide-binding protein superfamily, encodes a diadenosine polyphosphate (ApnA) hydrolase that cleaves substrates such as Ap3A to AMP plus the other nucleotide (Huebner *et al.*, 1998) and the hydrolytic activity is lost when His96 is replaced with Asn (Barnes *et al.*, 1996). This enzymatically inactive Fhit bound substrate well, yet still induced caspase-dependent apoptosis in cancer cells (Trapasso *et al.*, 2003), indicating that Ap3A binding activity was necessary for Fhit proapoptotic function, and prompting the hypothesis that the Fhit-substrate complex was the tumor suppressor signaler. It is also known that Fhit regulates the intracellular concentration of diadenosine triphosphate but not diadenosine tetraphosphate (Murphy *et al.*, 2000), suggesting that the *in vitro* defined activity of Fhit also occurs *in vivo*.

The human FHIT locus

At the time of identification of the FHIT gene, it was evident that the 1.7 Mb gene encompassed the most active, or most fragile of the human common chromosomal fragile sites, FRA3B at chromosome region 3p14.2 (Ohta *et al.*, 1996). Common fragile sites are large, evolutionarily conserved, unstable regions of human, mouse and other mammalian genomes, that are highly sensitive to genotoxic stress and perturbation of replication (summarized and cited in Durkin *et al.*, 2008). The molecular basis for genotoxic damage sensitivity and instability is not well understood, in spite of numerous hypotheses and investigations (reviewed in Pichiorri *et al.*, 2008). LeBeau and co-workers hypothesized that a unique epigenetic pattern might be involved and examined chromatin modification patterns at the six most highly recombinogenic human fragile sites and surrounding non-fragile regions (Jiang *et al.*, 2009). Chromatin at most of the fragile sites exhibited less histone acetylation than surrounding non-fragile regions, and treatment with DNA demethylating agent or an inhibitor of histone deacetylation reduced chromosome breakage at the fragile loci. There is now general agreement concerning the extreme sensitivity of fragile regions to DNA replication stress, resulting in very frequent alterations to the more active fragile loci in cancers. The FHIT and WWOX genes at the most frequently activated human fragile sites are also the most frequently altered genes in cancers, showing deletions, translocations, and promoter methylation in the majority of cancers. From detailed analyses of cancer-derived cell lines, many of the deletions are known to be identical in each cell of a derived cancer cell line, confirming clonal expansion, whether in the originating cancer or the derived cell line is not necessarily known. An outstanding question is: are these regions so frequently deleted in cancers because they are most sensitive to DNA damaging agents, or does the deletion drive the expansion of the preneoplastic cell in which damage occurred, or do both mechanisms contribute?

Role in oxidative and replicative stress

Recently, using chemical cross-linkers and immunoprecipitation, a Fhit protein complex was identified that includes Hsp60 and Hsp10, which may mediate Fhit stability and mitochondrial localization, where Fhit binds and stabilizes ferredoxin reductase (Fdxr); when Fdxr is over-expressed, it can lead to production of reactive oxygen species (ROS) that induce apoptosis. Cancer cells expressing endogenous or exogenous Fhit, when exposed to peroxide, an oxidative stress, produce higher levels of apoptosis-inducing ROS than matched, Fhit-negative cells; the Fhit-negative cancer cells survive, carrying DNA damage (Trapasso *et al.*, 2008; Pichiorri *et al.*, 2009; for review, Saldivar *et al.*, 2010). In addition to this mitochondrial function, Fhit over-expression in cancer cells exposed to replicative stress-inducing agents leads to enhanced caspase 3 activation and apoptosis, due to defective Chk1 activation. Thus, damage to the fragile FHIT locus leads to reduced expression of Fhit protein, and makes a two-pronged contribution to development of preneoplastic clonal expansion: (1) absence or reduction of Fhit leads to reduced expression of Fdxr and reduced ROS-induced apoptosis; (2) cells that escape ROS- or replicative stress-induced apoptosis can carry misrepaired DNA damage. The aberrant DNA damage response checkpoint in Fhit-deficient preneoplasias and cancers may make these lesions targets for inhibitors of proteins such as Parp1 and Chk1 with important roles in checkpoint responses, as observed for BRCA1-deficient cancer cells that also exhibit DNA damage repair deficiencies (Okumura *et al.*, 2009 for review).

Role in DNA damage response

The FHIT gene encompasses the most active common fragile site of the human genome and is thus exquisitely sensitive to intragenic alterations by DNA damaging agents, alterations that can lead to FHIT allele loss very early in the preneoplastic phase of cancer development, before or coincident with activation of the DNA damage checkpoint. Fhit

protein expression is lost or reduced in many preneoplastic lesions and in >50% of cancers, Fhit knockout mice are highly susceptible to carcinogen induction of tumors and Fhit replacement in these mice by gene therapy induces apoptosis and significantly reduces tumor burden (Ishii *et al.*, 2001). The study of Fhit deficient mouse and human tissue-derived and cancer-derived cells *in vitro* has led to several important conclusions (Ishii *et al.*, 2007): repair protein-deficient cancers are more likely to be Fhit-deficient; Fhit-deficient cells show enhanced resistance to UVC, mitomycin C, camptothecin and IR-induced cell killing, possibly due to strong activation of the ATR pathway following DNA damage; Fhit-deficient cells show higher efficiency of homologous recombination repair, a double-strand break repair pathway in mammalian cells; Fhit protein indirectly affects S-phase checkpoint and DNA repair. These studies suggested that the DNA damage-susceptible FRA3B/FHIT chromosome fragile region, paradoxically, encodes a protein, Fhit, that is necessary for protecting cells from accumulation of DNA damage (Ishii *et al.*, 2006,2007; Hu *et al.*, 2005). Thus, inactivation of Fhit contributes to accumulation of abnormal checkpoint phenotypes in cancer development.

A brief summary of characteristics of Hit protein family members discussed above is given in Table 1.

Interactors

Throughout the 14-year period since Fhit discovery, there have been reports of Fhit-interacting proteins that should help to define specific roles of Fhit in specific signal pathways (see Table 2 for summary). We used the yeast 2-hybrid method to seek Fhit interactors and found a number of candidates, none of which we could confirm by co-immunoprecipitation experiments. (KH lab, unpublished data). Our collaborator, Larry Barnes, reported (Chaudhuri *et al.*, 1999) that both wild-type and mutated forms of Fhit bind to tubulin strongly and specifically; his experiments used purified bovine tubulin preparations and purified Fhit proteins. Fhit did not cause nucleation or formation of microtubules, but in the presence of microtubule-associated proteins promoted assembly, and the microtubules formed appeared normal by electron microscopy. This would be an interesting activity for Fhit but we were not able to observe an interaction by co-immunoprecipitation.

Nishizaki *et al.* (2004), in a report about the synergistic effects of exogenous expression of p53 and Fhit, noted that Fhit binds the p53-interacting protein, MDM2, another interaction that would provide clues to how Fhit functions as a tumor suppressor, but this interaction was not confirmed by co-immunoprecipitation experiments (Y. Pekarsky, personal communication).

Shi *et al.* (2000) also used the yeast 2-hybrid system to seek Fhit interactors and reported that UBC9 protein is specifically associated with Fhit, an association that they report was confirmed in both immunoprecipitation and glutathione S-transferase pull-down assays. Given that yeast UBC9 is involved in the degradation of S- and M-phase cyclins, the authors suggested that Fhit may be involved in cell cycle control through its interaction with hUBC9. Again, this would have been an exciting function for Fhit; we had also observed UBC9-Fhit interaction in our yeast 2-hybrid experiments but have not been able to confirm this interaction by co-immunoprecipitation.

Finally, interaction of both Hint1 (Weiske & Huber, 2005) and Fhit (Weiske *et al.*, 2007) with beta-catenin (Bcat) have been reported. Bcat has an important role in the Wnt pathway that is deregulated in many types of cancer, so this would be a very interesting interaction for Fhit. The authors found that by binding to the Bcat C-terminal domain, Fhit repressed transcription of target genes such as cyclin D1 and survivin, and Fhit knockdown reversed

this effect. Fhit diadenosine-polyposphate hydrolase activity was not required for the down-regulation of Bcat-mediated transcription. Chromatin immunoprecipitation experiments were used to show recruitment of Fhit/Bcat complexes to target gene promoters, which would require at least a partial localization of Fhit in cell nuclei. Although we have not yet observed nuclear localization of Fhit in MCF7 breast cancer cells (see Fig. 2), it is possible that Fhit could localize to the nucleus under specific conditions. Thus, we will continue to look for confirmation of a possible role for Fhit in the Bcat pathway.

Nit1/2

In seeking to learn more about Fhit function, FHIT gene homologs were cloned from *D. melanogaster* and from *C. elegans* and the results showed that both genes encode fusion proteins with the Fhit domain fused to an N-terminal domain homologous to bacterial and plant nitrilases; the fly fusion protein exhibited diadenosine triphosphate hydrolase activity as expected for a Fhit homolog (Pekarsky *et al.*, 1998). In human and mouse, the nitrilase homologs and Fhit are encoded by two different genes on different chromosomes. The human and murine NIT1 genes were then cloned, exon-intron structure and expression pattern determined. Because fusion proteins with dual activities have been found to carry out specific steps in a biochemical or biosynthetic pathway, it was supposed that Fhit and Nit1 might collaborate in a biochemical or cellular pathway in mammalian cells. This suggestion has not yet been shown to be true, partially because the biological substrate for the mammalian gene designated as Nit1, which is likely not a nitrilase, has not been discovered, in spite of many attempts (C. Brenner, unpublished results).

The *C. elegans* NitFhit protein has been purified, crystallized and its structure determined (Pace *et al.*, 2000). Nit monomers possess an alpha-beta-beta-alpha fold with a presumptive Cys-Glu-Lys catalytic triad and the monomers assemble into an elegant tetrameric structure with Fhit dimers at opposite poles.

Nit1 knockout mice are viable, fertile and long-lived and have been examined for phenotypes resembling the Fhit knockout phenotypes (Semba *et al.*, 2006): 1) Nit1 expression was observed in most normal tissues and overlapped partially with Fhit expression; 2) Nit1-deficient mouse kidney cells exhibited accelerated proliferation, resistance to DNA damage stress, and increased cyclin D1 expression; 3) cyclin D1 was up-regulated in Nit1 null mammary gland and skin; 4) Nit1 over-expression induced caspase-dependent apoptosis *in vitro*; and 5) Nit1 allele deficiency led to increased incidence of carcinogen-induced forestomach tumors. Thus, the biological effects of loss of Nit1 expression are similar to effects of Fhit loss. Expression of recombinant NIT1 and FHIT induced apoptosis in Fhit- and Nit1-deficient cells, respectively, suggesting that Nit1-Fhit interaction is not essential for function of either protein. The results suggest that Nit1 and Fhit participate in similar tumor suppressor signaling pathways. Tumor susceptibility of mice deficient for both Nit1 and Fhit develop more spontaneous and carcinogen-induced tumors than Fhit^{-/-} mice, suggesting that the extent of tumor susceptibility due to Nit1 and Fhit deficiency is additive, and that Nit1 and Fhit affect distinct signal pathways in mammals (Sun *et al.*, 2009). Nit1, like Fhit, is present in cytoplasm and mitochondria but not nuclei.

Several branches of the nitrilase superfamily are found in mammalian genomes. The vanins/biotinidases/pantetheinases are involved in vitamin synthesis and the glutaminase domains of glutamine-dependent NAD synthases generate ammonia from glutamine for NAD synthesis. β -ureidopropionases (Up β) catalyze the hydrolysis of N-carbamoyl β -alanine (Car β Ala) to β -alanine, which is a central step in the pyrimidine catabolic pathway. Mutation of Up β in humans leads to a buildup of Car β Ala, which exerts neurotoxic effects

resulting in severe neuropathology and movement disorders (cited in Pace & Brenner, 2001 review).

As cited by Barglow *et al.*, (2008) when reporting crystal structure of Nit2, all mammalian species contain NIT1 and NIT2 genes, which share ~40% sequence identity. Over-expression of Nit2 in HeLa cells leads to a decrease in cell proliferation and growth, through induction of G2 arrest, indicating that Nit2 may also play a role in tumor suppression. Nit1 and Nit2 show distinct tissue distributions, with Nit2 highly expressed in liver and kidney, as well as fertilized eggs and oocytes, and Nit1 showing more ubiquitous expression across mouse tissues. Barglow *et al.*, (2008) have determined the crystal structure of mouse Nit2, a dimer, which is quite different from the tetrameric structure of the Nit1 domain of the worm NitFhit protein. The active site of mouse Nit2 closely resembles that of the Nit domain of NitFhit.

Three recent studies have identified Nit2 as omega-Amidase (omega-amidodicarboxylate amidohydrolase, EC 3.5.1.3) (Krasnikov *et al.*, 2009a,b; Jaisson *et al.*, 2009), a versatile enzyme that catalyzes amidase, transamidase, and ester hydrolysis reactions. omega-Amidase activity toward alpha-ketoglutaramate and alpha-ketosuccinamate (the alpha-keto acid analogues of glutamine and asparagine, respectively) is present in mammalian tissues, tumors, plants, bacteria, and fungi. omega-Amidase has recently been shown to be identical to Nit2, a putative tumor suppressor. Identification of the substrate of Nit2 may help to characterize the function of omega-Amidase/Nit2 in tumor suppression and assist in identifying biological interactions between nitrogen metabolism and tumor biology. For example, some evidence suggests that the substrate, alpha-ketoglutaramate, may be neurotoxic. Thus, an important role of omega-Amidase may be to remove potentially toxic intermediates by converting alpha-ketoglutaramate and alpha-ketosuccinamate to biologically useful alpha-ketoglutarate and oxaloacetate, respectively.

Summary

We have briefly summarized what is known about these proteins, but in closing wish to feature the outstanding questions.

Hint1 was discovered mistakenly as an inhibitor of Protein Kinase C and designated Pkci, a designation that still confuses the literature. The other Hint family members were discovered by homology to Hint1. Aprataxin was discovered as a result of the hunt for a gene responsible for AOA1. Fhit was discovered through cloning of a familial chromosome translocation breakpoint on chromosome 3 that interrupts the large FHIT gene within an intron, in the FRA3B chromosome region (Ohta *et al.*, 1996), now known to be the region of the human genome most susceptible to DNA damage due to replication stress (Durkin *et al.*, 2008). The NitFhit fusion gene was discovered during searches for Fhit homologs in flies and worms because the fly/worm Nit polypeptide is fused to the 5'-end of the Fhit gene; the mammalian Nit gene family was discovered because of the NitFhit fusion gene, in searches for homologs to the Nit polypeptide of the NitFhit gene. Each of the Hit family member proteins is reported to have enzymatic activities toward putative substrates involving nucleosides or dinucleosides. Most surprisingly, each of the Hit family proteins discussed has been implicated in important DNA damage response pathways and/or tumor suppression pathways. And for each of them it has been difficult to assign definite substrates, to know if the substrates and catalytic products have biological functions, to know if that function is related to the DNA damage response and suppressor functions, and to precisely define the pathways through which tumor suppression occurs.

When the fly Nit sequence was found at the 5'-end of the fly Fhit gene, this gene was hailed as a Rosetta stone gene/protein that would help in discovery of the function of Fhit, because the Nit protein should be in the same signal pathway (Pace *et al.*, 2000). However, the

mammalian Nit family proteins have turned out to be at least as mysterious as the Fhit proteins, with the Nit1 substrate still unknown and the surprising finding that Nit proteins also appear to behave as tumor suppressor proteins. Whether the predicted enzymatic functions of these proteins are relevant to the observed biological functions, remain among the outstanding unanswered puzzles and raise the question: have these mammalian proteins evolved beyond the putative original enzymatic purpose, such that the catalytic function is now vestigial and subservient to signal pathways that use the protein substrate complexes in pathways that signal apoptosis or DNA damage response? Or can these proteins be fulfilling catalytic functions independently but in parallel with signal pathway functions, as perhaps observed for Aprataxin? Or is the catalytic function indeed part of the observed biological functions, such as apoptosis and tumor suppression? Perhaps the recent, post-genomic focus on metabolomics and genome-wide investigations of signal pathway networks will lead to answers to some of these outstanding questions.

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References

- Barglow KT, Saikatendu KS, Bracey MH, Huey R, Morris GM, Olson AJ, Stevens RC, Cravatt BF. Functional proteomic and structural insights into molecular recognition in the nitrilase family enzymes. *Biochemistry*. 2008; 47:13514–13523. [PubMed: 19053248]
- Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, Croce CM, Ohta M, Huebner K. Fhit, a putative tumor suppressor in humans, is a dinucleoside 5,5-P1,P3-triphosphate hydrolase. *Biochemistry*. 1996; 35:11529–11535. [PubMed: 8794732]
- Bieganski P, Garrison PN, Hodawadekar SC, Faye G, Barnes LD, Brenner C. Adenosine monophosphoramidase activity of Hint and Hnt1 supports function of Kin28, Ccl1, and Tfb3. *J Biol Chem*. 2002; 277:10852–10860. [PubMed: 11805111]
- Brenner C. Hint, Fhit, and GalT: Function, structure, evolution, and mechanism of three branches of the histidine triad superfamily of nucleotide hydrolases and transferases. *Biochemistry*. 2002; 41:9003–9014. [PubMed: 12119013]
- Brenner C, Bieganski P, Pace HC, Huebner K. The histidine triad superfamily of nucleotide-binding proteins. *J Cell Physiol*. 1999; 181:179–187. [PubMed: 10497298]
- Cen B, Li H, Weinstein IB. Histidine triad nucleotide-binding protein 1 up-regulates cellular levels of p27KIP1 by targeting ScfSKP2 ubiquitin ligase and Src. *J Biol Chem*. 2009; 284:5265–5276. [PubMed: 19112177]
- Chaudhuri AR, Khan IA, Prasad V, Robinson AK, Ludueña RF, Barnes LD. The tumor suppressor protein Fhit. A novel interaction with tubulin. *J Biol Chem*. 1999; 274:24378–24382. [PubMed: 10446217]
- Chou TF, Baraniak J, Kaczmarek R, Zhou X, Cheng J, Ghosh B, Wagner CR. Phosphoramidate pronucleotides: a comparison of the phosphoramidase substrate specificity of human and *Escherichia coli* histidine triad nucleotide binding proteins. *Mol Pharm*. 2007; 4:208–217. [PubMed: 17217311]
- Date H, Onodera O, Tanaka H, Iwabuchi K, Uekawa K, Igarashi S, Koike R, Hiroi T, Yuasa T, Awaya Y, Sakai T, Takahashi T, Nagatomo H, Sekijima Y, Kawachi I, Takiyama Y, Nishizawa M, Fukuhara N, Saito K, Sugano S, Tsuji S. Early-onset ataxia with ocular motor apraxia and hypoalbuminemia is caused by mutations in a new HIT superfamily gene. *Nat Genet*. 2001; 29:184–188. [PubMed: 11586299]
- Durkin SG, Ragland RL, Arlt MF, Mulle JG, Warren ST, Glover TW. Replication stress induces tumor-like microdeletions in FHIT/FRA3B. *Proc Natl Acad Sci USA*. 2008; 105:246–251. [PubMed: 18162546]

- Fong LY, Fidanza V, Zanesi N, Lock LF, Siracusa LD, Mancini R, Siprashvili Z, Ottey M, Martin SE, Druck T, McCue PA, Croce CM, Huebner K. Muir-Torre-like syndrome in Fhit-deficient mice. *Proc Natl Acad Sci USA*. 2000; 97:4742–4747. [PubMed: 10758156]
- Hu B, Han S, Wang X, Ottey M, Dicker A, Huebner K, Wang Y. Involvement of the Fhit gene in the ionizing radiation-activated ATR/CHK1 pathway. *J Cell Physiol*. 2005; 202:518–523. [PubMed: 15389587]
- Huang Y, Garrison PN, Barnes LD. Cloning of the *Schizosaccharomyces pombe* gene encoding diadenosine 5',5'''-P₁P₄-tetrphosphate (Ap4A) asymmetrical hydrolase: sequence similarity with the histidine triad (HIT) protein family. *Biochem J*. 1995; 312:925–932. [PubMed: 8554540]
- Huebner K, Garrison PN, Barnes LD, Croce CM. The role of the FHIT/FRA3B locus in cancer. *Annu Rev Genet*. 1998; 32:7–31. Review. [PubMed: 9928473]
- Ingram SW, Barnes LD. Disruption and overexpression of the *Schizosaccharomyces pombe* aph1 gene and the effects on intracellular diadenosine 5',5'''-P₁, P₄-tetrphosphate (Ap4A), ATP and ADP concentrations. *Biochem J*. 2000; 350:663–669. [PubMed: 10970777]
- Ishii H, Dumon KR, Vecchione A, Fong LY, Baffa R, Huebner K, Croce CM. Potential cancer therapy with the fragile histidine triad gene: review of the preclinical studies. *JAMA*. 2001; 286:2441–2449. Review. [PubMed: 11712940]
- Ishii H, Mimori K, Inoue H, Inageta T, Ishikawa K, Semba S, Druck T, Trapasso F, Tani K, Vecchione A, Croce CM, Mori M, Huebner K. Fhit modulates the DNA damage checkpoint response. *Cancer Res*. 2006; 66:11287–11292. [PubMed: 17145874]
- Ishii H, Wang Y, Huebner K. A Fhit-ing role in the DNA damage checkpoint response. *Cell Cycle*. 2007; 6:1044–1048. [PubMed: 17457056]
- Jaisson S, Veiga-da-Cunha M, Van Schaftingen E. Molecular identification of omega-amidase, the enzyme that is functionally coupled with glutamine transaminases, as the putative tumor suppressor Nit2. *Biochimie*. 2009; 91:1066–1071. [PubMed: 19596042]
- Jiang Y, Lucas I, Young DJ, Davis EM, Karrison T, Rest JS, Le Beau MM. Common fragile sites are characterized by histone hypoacetylation. *Hum Mol Genet*. 2009; 18:4501–4512. [PubMed: 19717471]
- Kijas AW, Harris JL, Harris JM, Lavin MF. Aprataxin forms a discrete branch in the HIT (histidine triad) superfamily of proteins with both DNA/RNA binding and nucleotide hydrolase activities. *J Biol Chem*. 2006; 281:13939–13948. [PubMed: 16547001]
- Krasnikov BF, Chien CH, Nostramo R, Pinto JT, Nieves E, Callaway M, Sun J, Huebner K, Cooper AJ. Assay and purification of omega-amidase/Nit2, a ubiquitously expressed putative tumor suppressor, that catalyzes the deamidation of the alpha-keto acid analogues of glutamine and asparagine. *Biochimie*. 2009a; 91:1072–1080. [PubMed: 19595734]
- Krasnikov BF, Nostramo R, Pinto JT, Cooper AJ. Identification of the putative tumor suppressor Nit2 as omega-amidase, an enzyme. *Anal Biochem*. 2009b; 391:144–150. [PubMed: 19464248]
- Li H, Zhang Y, Su T, Santella RM, Weinstein IB. Hint1 is a haplo-insufficient tumor suppressor in mice. *Oncogene*. 2005; 25:713–721. [PubMed: 16186798]
- Li H, Balajee AS, Su T, Cen B, Hei TK, Weinstein IB. The HINT1 tumor suppressor regulates both gamma-H2AX and ATM in response to DNA damage. *J Cell Biol*. 2008; 183:253–265. [PubMed: 18852295]
- Lima CD, Klein MG, Weinstein IB, Hendrickson WA. Three-dimensional structure of human protein kinase C interacting protein 1, a member of the HIT family of proteins. *Proc Natl Acad Sci USA*. 1996; 93:5357–5362. [PubMed: 8643579]
- Martin J, Magnino F, Schmidt K, Piguet AC, Lee JS, Semela D, St-Pierre MV, Ziemiecki A, Cassio D, Brenner C, Thorgeirsson SS, Dufour JF. Hint2, a mitochondrial apoptotic sensitizer down-regulated in hepatocellular carcinoma. *Gastroenterology*. 2006; 130:2179–2188. [PubMed: 16762638]
- Moreira MC, Barbot C, Tachi N, Kozuka N, Uchida E, Gibson T, Mendonça P, Costa M, Barros J, Yanagisawa T, Watanabe M, Ikeda Y, Aoki M, Nagata T, Coutinho P, Sequeiros J, Koenig M. The gene mutated in ataxia-ocular apraxia 1 encodes the new HIT/Zn-finger protein aprataxin. *Nat Genet*. 2001; 29:189–193. [PubMed: 11586300]

- Murphy GA, Halliday D, McLennan AG. The Fhit tumor suppressor protein regulates the intracellular concentration of diadenosine triphosphate but not diadenosine tetraphosphate. *Cancer Res.* 2000; 60:2342–2344. [PubMed: 10811104]
- Nishizaki M, Sasaki J, Fang B, Atkinson EN, Minna JD, Roth JA, Ji L. Synergistic tumor suppression by coexpression of FHIT and p53 coincides with FHIT-mediated MDM2 inactivation and p53 stabilization in human non-small cell lung cancer cells. *Cancer Res.* 2004; 64:5745–5752. [PubMed: 15313915]
- Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Druck T, Croce CM, Huebner K. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell.* 1996; 84:587–597. [PubMed: 8598045]
- Okumura H, Ishii H, Pichiorri F, Croce CM, Mori M, Huebner K. Fragile gene product, Fhit, in oxidative and replicative stress responses. *Cancer Sci.* 2009; 100:1145–1150. [PubMed: 19486340]
- Pace HC, Garrison PN, Robinson AK, Barnes LD, Draganescu A, Rosler A, Blackburn GM, Siprashvili Z, Croce CM, Huebner K, Brenner C. Genetic, biochemical and crystallographic definition of a substrate analog complex with the fragile histidine triad protein as the active signaling form of Fhit. *Proc Natl Acad Sci USA.* 1998; 95:5484–5489. [PubMed: 9576908]
- Pace HC, Brenner C. The nitrilase superfamily: classification, structure and function. *Genome Biol.* 2001; 2 REVIEWS0001.
- Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganski P, Pekarsky Y, Croce CM, Brenner C. Crystal structure of the worm NitFhit Rosetta Stone protein reveals a Nit tetramer binding two Fhit dimers. *Curr Biol.* 2000; 10:907–917. [PubMed: 10959838]
- Pekarsky Y, Campiglio M, Siprashvili Z, Druck T, Sedkov Y, Tillib S, Draganescu A, Wermuth P, Rothman JH, Huebner K, Buchberg AM, Mazo A, Brenner C, Croce CM. Nitrilase and Fhit homologs are encoded as fusion proteins in *Drosophila melanogaster* and *Caenorhabditis elegans*. *Proc Natl Acad Sci USA.* 1998; 95:8744–8749. [PubMed: 9671749]
- Pichiorri F, Ishii H, Okumura H, Trapasso F, Wang Y, Huebner K. Molecular parameters of genome instability: roles of fragile genes at common fragile sites. *J Cell Biochem.* 2008; 104:1525–1533. [PubMed: 18393361]
- Pichiorri F, Okumura H, Nakamura T, Garrison PN, Gasparini P, Suh SS, Druck T, McCorkell KA, Barnes LD, Croce CM, Huebner K. Correlation of fragile histidine triad (Fhit) protein structural features with effector interactions and biological functions. *J Biol Chem.* 2009; 284:1040–1049. [PubMed: 19004824]
- Reynolds JJ, El-Khamisy SF, Caldecott KW. Short-patch single-strand break repair in ataxia oculomotor apraxia-1. *Biochem Soc Trans.* 2009a; 37:577–581. [PubMed: 19442253]
- Reynolds JJ, El-Khamisy SF, Katyal S, Clements P, McKinnon PJ, Caldecott KW. Defective DNA ligation during short-patch single-strand break repair in ataxia oculomotor apraxia 1. *Mol Cell Biol.* 2009b; 29:1354–1362. [PubMed: 19103743]
- Saldivar JC, Shibata H, Huebner K. Pathology and biology associated with the fragile FHIT gene and gene product. *J Cell Biochem.* 2010; 109:858–865. [PubMed: 20082323]
- Semba S, Han SY, Qin HR, McCorkell KA, Iliopoulos D, Pekarsky Y, Druck T, Trapasso F, Croce CM, Huebner K. Biological functions of mammalian Nit1, the counterpart of the invertebrate NitFhit Rosetta stone protein, a possible tumor suppressor. *J Biol Chem.* 2006; 281:28244–28253. [PubMed: 16864578]
- Shi Y, Zou M, Farid NR, Paterson MC. Association of FHIT (fragile histidine triad), a candidate tumour suppressor gene, with the ubiquitin-conjugating enzyme hUBC9. *Biochem J.* 2000; 352:443–448. [PubMed: 11085938]
- Siprashvili Z, Sozzi G, Barnes LD, McCue P, Robinson AK, Eryomin V, Sard L, Tagliabue E, Greco A, Fusetti L, Schwartz G, Pierotti MA, Croce CM, Huebner K. Replacement of Fhit in cancer cells suppresses tumorigenicity. *Proc Natl Acad Sci USA.* 1997; 94:13771–13776. [PubMed: 9391102]
- Sun J, Okumura H, Yearsley M, Frankel W, Fong LY, Druck T, Huebner K. Nit1 and Fhit tumor suppressor activities are additive. *J Cell Biochem.* 2009; 107:1097–1106. [PubMed: 19479888]

- Trapasso F, Krakowiak A, Cesari R, Arkles J, Yendamuri S, Ishii H, Vecchione A, Kuroki T, Bieganski P, Pace HC, Huebner K, Croce CM, Brenner C. Designed FHIT alleles established that Fhit-induced apoptosis in cancer cells is limited by substrate-binding. *Proc Natl Acad Sci USA*. 2003; 100:1592–1597. [PubMed: 12574506]
- Trapasso F, Pichiorri F, Gasparo M, Palumbo T, Aqeilan RI, Gaudio E, Okumura H, Iuliano R, Okumura H, Di Leva G, Fabbri M, Birk DE, Raso C, Green-Church K, Spagnoli LG, Venuta S, Huebner K, Croce CM. Fhit interaction with ferredoxin reductase triggers generation of reactive oxygen species and apoptosis. *J Biol Chem*. 2008; 283:13736–13744. [PubMed: 18319262]
- Weiske J, Huber O. The histidine triad protein Hint1 interacts with Pontin and Reptin and inhibits TCF-beta-catenin-mediated transcription. *J Cell Sci*. 2005; 118:3117–3129. [PubMed: 16014379]
- Weiske J, Albring KF, Huber O. The tumor suppressor Fhit acts as a repressor of beta-catenin transcriptional activity. *Proc Natl Acad Sci USA*. 2007; 104:20344–20349. [PubMed: 18077326]
- Zanesi N, Fidanza V, Fong LY, Mancini R, Druck T, Valtieri M, Rüdiger T, McCue PA, Croce CM, Huebner K. The tumor spectrum in FHIT-deficient mice. *Proc Natl Acad Sci USA*. 2001; 98:10250–10255. [PubMed: 11517343]

Fig. 1. Alignment of amino acid sequences to show the relationships among Hint, Fhit and Nit proteins

Human Hint1 and 2 (red), *C. elegans* and *D. melanogaster* NitFhit (blue/black), human Fhit (black) and human Nit1 and 2 (blue) protein sequences were aligned using ClustalW. The most highly conserved amino acids are highlighted yellow (Nit specific) or green (Fhit specific). The signature, conserved Cys and His are starred for Nits and Fhits/Hints, respectively.

Fig. 2. Subcellular localization of Fhit in MCF7 breast cancer cells

After isolation of nuclear and cytoplasmic cellular fractions from MCF7 breast cancer cells expressing abundant Fhit, 50 μ g protein was loaded to each lane of the gel, proteins were transferred and assessed by immunoblot for expression of Fhit in specific subcellular fractions. GAPDH was used as a cytosolic marker and PARP as nuclear marker.

Table 1

Characteristics of mammalian Hit family proteins

Protein	Substrate	Interactors	Sublocalization	Biological Function
Hint1	AMP-NH ₂ , AMP-Lys	SCF ubiquitin ligase	nuclear after IR	Stability of p27, tumor suppression
Hint2	AMP-NH ₂ , AMP-Lys?		mitochondrial	apoptosis
Aprataxin	5' AMP-DNA?	XRCC4, PARP1, p53	nuclear	DNA damage response; mutations in Hit domain cause AOA1
Fhit	Ap ₃ A, Ap ₄ A	HSP60/10, Fdxr	cytosol, mitochondria	through Fdxr interaction induces ROS; participates in DNA damage response; tumor suppression

Table 2

Candidate Fhit interacting proteins

Candidate interactor	Method used to illustrate	Confirmed?	References
Tubulin	<i>In vitro</i> binding of purified proteins	Not detected by IP (Druck, Barnes, Huebner, unpublished)	Chaudhuri <i>et al.</i> , 1999
MDM2	Co-immunoprecipitation	Not detected by IP (Y. Pekarsky, personal communication)	Nishizaki <i>et al.</i> , 2004
UBC9	Co-immunoprecipitation	Not confirmed by us	Shi <i>et al.</i> , 2000
Bcat	Co-immunoprecipitation and sublocalization	Not confirmed by us	Weiske <i>et al.</i> , 2007
HSP60/10, Fdxr	Co-immunoprecipitation	No report of confirmation by others	Trapasso <i>et al.</i> , 2008; Pichiorri <i>et al.</i> , 2009