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Review

Hit proteins, mitochondria and cancer ☆

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ABSTRACT

The histidine triad (HIT) superfamily comprises proteins that share the histidine triad motif, His-ϕ-His-ϕ-His-ϕ–ϕ, where ϕ is a hydrophobic amino acid. HIT proteins are ubiquitous in prokaryotes and eukaryotes. HIT proteins bind nucleotides and exert dinucleotidyl hydrolase, nucleotidylyl transferase or phosphoramidate hydrolase enzymatic activity. In humans, 5 families of HIT proteins are recognized. The accumulated epidemiological and experimental evidence indicates that two branches of the superfamily, the HINT (Histidine Triad Nucleotide Binding) members and FHIT (Fragile Histidine Triad), have tumor suppressor properties but a conclusive physiological role can still not be assigned to these proteins. Aprataxin forms another discrete branch of the HIT superfamily, is implicated in DNA repair mechanisms and unlike the HINT and FHIT members, a defective protein can be conclusively linked to a disease, ataxia with oculomotor apraxia type 1. The scavenger mRNA decapping enzyme, DcpS, forms a fourth branch of the HIT superfamily. Finally, the GalT enzymes, which exert specific nucleoside monophosphate transferase activity, form a fifth branch that is not implicated in tumorigenesis. The molecular mechanisms by which the HINT and FHIT proteins participate in bioenergetics of cancer are just beginning to be unraveled. Their purported actions as tumor suppressors are highlighted in this review. This article is part of a Special Issue entitled: Bioenergetics of Cancer.

1. HIT proteins

Proteins containing the HIT domain constitute a superfamily of enzymes that share the motif, His-ϕ-His-ϕ-His-ϕ–ϕ, where ϕ is a hydrophobic amino acid. When considered according to their enzymatic activity, HIT proteins are classified as nucleoside phosphoramidate hydrolases, dinucleotide hydrolases or nucleotidylyl transferases. HIT proteins bind nucleotides in such a way that the His-ϕ-His-ϕ-His-ϕ–ϕ active site is positioned vis-à-vis the α-phosphate of the substrate. The key difference between the HIT hydrolase and the transferase activity is that the hydrolases do not require a second substrate to accept the histidine bound nucleotide but are able to transfer the nucleotide to water [1]. Mutation of the second His in the histidine triad abolishes the hydrolase activity of HIT proteins [2].

The HIT proteins have been conserved throughout evolution, which implies that they fulfill basic, perhaps vital functions [3]. More than 35 members of the HIT superfamily have been identified in 29 species including bacteria, archae, yeast, plants, *C. elegans*, Drosophila and mammals. The human genome encodes 7 HIT proteins, which can be classified into 5 branches (Fig. 4) [4].

2. HINT proteins (HIstidine Triad NucleoTide-binding proteins)

HINT (nomenclature: human HINT, non-human Hint) proteins constitute the first branch of the HIT superfamily. At least one HINT/Hint is present in all fully sequenced genomes. The human genome contains 3 separate genes that encode the HINT1, HINT2 and HINT3 gene products.

2.1. HINT1

HINT1 (HIstidine triad NucleoTide-binding protein 1), a 126 amino acid (Fig. 1), cytosolic protein, was first reported in 1990 to be a protein kinase C inhibitor [5] and appears in early literature as Protein Kinase C Inhibitor-1 (PKCI-1) [6–18]. The PKC inhibitory role is now viewed with skepticism although HINT1 may interact directly or indirectly with PKC [19] and therefore PKCI-1 was renamed HIstidine triad NucleoTide-binding protein 1 [20]. According to structural studies by crystallography and NMR spectroscopy HINT1 is a purine nucleotide-binding protein [20–22]. HINT1 forms homodimers and each subunit binds a nucleotide.

Brenner and coworkers discovered that HINT1 can hydrolyze adenosine 5′-monophosphoramidate (AMP-NH_2), an intracellular solute that is synthesized from AMP-SO_4 and ammonia in many eukaryotic organisms [2,23,24]. This monophosphoramidase activity is dependent upon the second His residue of the HIT domain since a His→Ala mutation abolishes this enzymatic activity [2]. The physiological importance of AMP-NH_2 is unknown and it is perhaps not the natural substrate of HINT1. HINT1 can hydrolyze AMP adducts bound to lysine residues of

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HINT1  ---------------------------------MADEIAKAQVARP-GGD--TIFGKIIRKEIP--AKIIFEDDRCLAFHDISPQAPTHFLVIPKKHISQISVAE
HINT2  MAAAVVLAAGLRAARRAVAATGVRGGQVRGAAGVTDGNEVAKAQQATP-GGAAPTIFSRILDKSLP--ADILYEDQQCLVFRDVAPQAPVHFLVIPKKPIPRISQAE
HINT3  -------MAEEQVNRSAGLAPDCEASATAETTVSSVGTCEAAGKSPEPKDYDSTCVFCRIAGRQDPGTELLHCENEDLICFKDIKPAATHHYLVVPKKHIGNCRTLR
FHIT   ----------------------------------------------------MSFRFGQHLIKPSVVFLKTELSFALVNRKPVVPGHVLVCPLRPVERFHDLR

HINT1 DDDESLLGHLMIVGKKCAADLGLNKGYRMVVNEGSDGGQSVYHVHLHVLG--GRQMHWPPG---------------------------------- 126
HINT2 EEDQQLLGHLLLVAKQTAKAEGLGDGYRLVINDGKLGAQSVYHLHIHVLG--GRQLQWPPG---------------------------------- 163
HINT3 KDQVELVENMVTVGKTILERNNFTDFTNVRMGFHMPPFCSISHLHLHVLAP-VDQLGFLSKLVYRVNSY--------WFITADHLIEKLRT----- 182
FHIT  PDEVADLFQTTQRVGTVVEKHFHGTSLTFSMQDGPEAGQTVKHVHVHVLPRKAGDFHRNDSIYEELQKHDKEDFPASWRSEEEMAAEAAALRVYFQ 147
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Fig. 1. Alignment of the amino acid sequences of human HINT1, HINT2, HINT3 and FHIT proteins. The histidine triad motif (His-φ-His-φ-His-φ–φ) is indicated (arrow) and mediates the nucleotide binding. The middle His of the motif is essential for hydrolase activity. The HINT1/HINT2/HINT3 proteins and the FHIT protein belong to two separate branches of the HIT superfamily. The Tyr at position 114 of FHIT (red arrow) is required for pro-apoptotic activity.

adenylylated proteins and therefore may regulate the nucleotidylation of protein substrates [1]. Chou et al. investigated the substrate specificity of HINT1 using a sensitive, continuous fluorescence-based assay to measure the phosphoramidate hydrolase activity. HINT1 showed a clear preference for purine over pyrimidine phosphoramidates [25].

Using purified lysyl-tRNA synthetase, Chou and Wagner identified lysyl-AMP as a substrate for HINT1, which yields an adenylated form of the enzyme [26]. This finding has focused attention on the contribution that HINT1 could make to a variety of cellular functions. Aminoacyl-tRNA synthetases are the essential enzymes that charge amino acids with their corresponding cognate tRNAs to form aminoacyl-tRNAs. The reaction proceeds by the formation of an aminoacyl-AMP intermediate. Aminoacyl-tRNA synthetases have pleiotropic physiological roles in transcription, translation, splicing, inflammation, angiogenesis and apoptosis [27]. Several aminoacyl-tRNA synthetases (E, I, L, R, Q, M, K, D) associate with three aminoacyl-tRNA synthetase-interacting multi-functional proteins (AIMP), AIMP1/p43, AIMP2/p38 and AIMP3/p18 to form macromolecular complexes. AIMP1 is a precursor of endothelial monocyte-activating polypeptide-II, is secreted as a proinflammatory cytokine and increases proliferation and collagen production in fibroblasts, induces migration of endothelial cells and activates macrophages [28]. AIMP2 is a proapoptotic factor that interacts with p53 [29], and AIMP3 is a tumor suppressor that activates repair of damaged DNA [27]. Lysyl-tRNA synthetase is required to lend stability to the complex [30]. Surprisingly, human lysyl-tRNA synthetase was found to be secreted from various cell lines and to interact with cell surface chemokine receptors to stimulate the formation of TNFalpha. Tryptophanyl and tyrosyl-tRNA synthetases are also procytokines but are not generally associated with the complex [31,32].

Most of the experimental evidence supports a role for HINT1 in the regulation of transcription that could affect tumorigenesis signaling pathways. Razin and coworkers documented a functional multiprotein interaction between Hint1, lysyl-tRNA synthetase and the microthalmia transcription factor (MITF), a melanoma oncogene whose expression transforms primary human melanocytes and increases their chemo-resistance [33], as well as with the upstream stimulatory factor 2 (USF2), an oncogene ubiquitously expressed in eukaryotic cells [34,35]. Besides its function in the aminoacylation of tRNA, lysyl-tRNA synthetase produces the signaling molecule, diadenosine tetraphosphate (Ap_4A) [36]. Both MITF and USF2 are inhibited when associated with HINT1. However, Ap4A can bind to HINT1 and provoke the dissociation of HINT1 from MITF or USF2 and allow trans-activation (Fig. 2). HINT1 was associated physically with the basal transcription factor TFIIH via an interaction with the cyclin dependent kinase Cdk7 [2,11]. A similar interaction was reported in *S. cerevisiae*, between Hnt1 and Kin28, which are the yeast orthologues

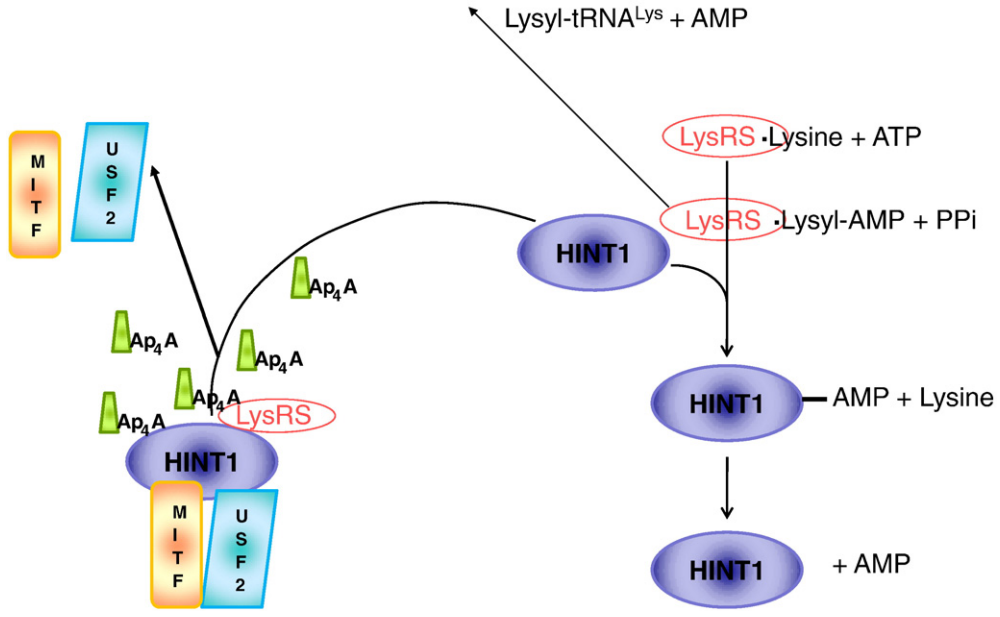

Fig. 2. Proposed mechanism of adenylation of HINT1. HINT1 can associate with and thereby inhibit transcription factors such as microphthalmia transcription factor (MITF) and upstream stimulatory factor 2 (USF2) in a complex with lysyl-tRNA synthetase (LysRS). Lys RS synthesizes aminoacyl-tRNA via an aminoacyl-AMP intermediate and also catalyzes the formation of Ap_4A. HINT1 is dissociated from the complex by binding to Ap_4A. The His residue of the HIT domain binds to the substrate, lysyl-AMP generated by LysRS, to form an adenylated enzyme intermediate. HINT1 hydrolyzes lysyl-AMP.

of HINT1 and Cdk7, respectively. The genetic disruption of Hnt1 and Kin28 resulted in elongated cells and reduced colony formation, a phenotype not apparent after disruption of Hnt1 alone [11]. However, studies in Hint1$^{-/-}$ mice failed to show a phenotype consistent with a role for Hint1 in the regulation of Cdk7 activity [37]. Weiske and Huber identified an interaction between HINT1 and the β-catenin partners, pontin and reptin. In functional experiments, HINT1 acted as a negative regulator of TCF-β-catenin transcriptional activity, thereby repressing the expression of target genes of the Wnt signaling pathway such as axin2 and cyclin D1 [18]. In pull-down assays, HINT1 did not bind to β-catenin or LEF-1 nor did it impair the LEF-1/β-catenin interaction or the binding of pontin or reptin to β-catenin. Rather, HINT1 disrupted pontin/reptin complexes and was able to bind to the histone acetylase Tip60 [38]. This suggests that HINT1/Tip60 complexes have a repressive function at least on a subset of TCF/β-catenin target genes.

Transient transfection of HINT1 into SW480 and MCF-7 cells induced apoptosis associated with increased expression of p53, Bax and decreased expression of Bcl-2. The silencing of HINT1 down-regulated the expression of p53 and Bax. HINT1 was retrieved from the Bax promoter in a complex with Tip60 [39].

Su et al. reported that mice lacking Hint1 developed more tumors when treated with a carcinogen [17]. Not only Hint1$^{-/-}$ mice, but also Hint1$^{+/-}$ mice were more susceptible to the induction of mammary and ovarian tumors than wild-type mice [40]. In an effort to elucidate mechanisms of tumor suppression, Weinstein's group discovered that Hint1 binds to the protein 'plenty of SH3 domains' (POSH), a scaffold protein that participates in a multimeric complex with Rac-1, mixed lineage kinase 3(MLK3), MAPK kinase 4/7 and JNK1/2 [41,42]. POSH increases JNK activation and apoptosis and its ectopic expression stimulates nuclear translocation of NF-κB [43]. The overexpression of POSH promotes neuronal apoptosis and the silencing of POSH with antisense oligonucleotides and siRNA suppresses JNK activity and neuronal apoptosis induced by NGF withdrawal [44] and prevents ischemic injury by inhibiting the MLK3-MAPK kinase 4-JNK signalling pathway and caspase 3 activation [45]. Apoptotic stimuli increase endogenous cellular levels of POSH and MLKs, an effect that occurs through protein stabilization [46]. The Hint1/POSH interaction impairs the ability of JNK2 to phosphorylate activator protein 1 (AP-1), which is an important transcription factor in cancer cells [41]. In addition to inhibiting AP-1 transcription activity, HINT1 also interacts with and suppresses the S-phase-kinase-associated protein 2 (SKP2) SCF ubiquitin ligase complex, which regulates the ubiquitination of the cell cycle regulator, p27^{KIP1} [47]. The particular importance of HINT1 in hepatocellular carcinoma has garnered special attention along with other tumor suppressors implicated in liver carcinogenesis. The promoter of HINT1 is frequently hypermethylated in hepatocellular carcinoma, and this is associated with a lower expression of HINT1 in tumor tissue [48]. HINT1 can thus be added to the list of tumor suppressor genes subject to DNA promoter methylation as a means of transcriptional inactivation [49]. The extent of HINT1 hypermethylation was even correlated with the prognosis of HCC [50]. The poorer the prognosis of HCC, the greater the extent of gene hypermethylation and the lower the expression level of HINT1 protein. The findings of Calvisi et al. imply that HINT1 influences the activity of SKP2, which in turn yields more degradation of the cell cycle regulator, p27^{KIP1}, all features of HCC with a poor prognosis. An additional discovery that likely influences the tumor suppressor properties of HINT1, is its participation in repair of DNA double strand breaks [51]. Mouse Hint1 co-immunoprecipitated with γ-Histone 2AX and ATM (ataxia-telangiectasia mutated) protein kinase after ionizing radiation-induced DNA damage, an event that normally activates and recruits both proteins to the focus of DNA breaks. Hint1$^{-/-}$ mouse embryonic fibroblasts are more resistant to cytotoxicity and apoptosis after ionizing radiation [17], presumably because Hint1 can no longer regulate the functions of γ-Histone 2AX and ATM [51].

Evidence of biological functions of HINT1 in neurons was recently uncovered. The C terminus of the μ-opioid receptor binds to HINT1, which in turn binds to another protein, RGS-z, a member of the family of regulator of G-protein signaling (RGS) proteins. Cerebroventricular administration of morphine recruits PKC isoforms to the μ-opioid receptor via a HINT1/RGS-z complex. When HINT1 expression was lowered, morphine produced stronger analgesic effects and neither the PKCγ- μ-opioid receptor complex nor serine phosphorylation of this receptor was detected [52]. Microarray studies reported decreased mRNA expression of HINT1 in the frontal cortex of individuals with schizophrenia, suggesting a link to the pathophysiology of the disease [53,54]. Consistent with the documented link between dopamine transmission and schizophrenia, systemic administration of the direct-acting dopamine receptor agonist apomorphine significantly increased locomotor activity in Hint1$^{-/-}$ mice in contrast to control mice [55]. Moreover, apomorphine elicited anxiolytic and anti-depressant like properties in Hint1$^{-/-}$ mice [55].

Despite the ongoing productive research in the field, the authentic function of HINT1 in cellular physiology remains elusive. The specific residues engaged in the binding of nucleotides are those conserved residues in the domain shared by the HIT families [22], which supports the notion that nucleotide binding is an inextricable part of HINT1's function. Unlike its binding to nucleotides, the binding of HINT1 to other protein partners has not gathered the same degree of structural scrutiny. Identifying the structural motifs that mediate HINT1–protein interactions and determining whether nucleotidyl hydrolase activity ever factors into its tumor suppressor properties are outstanding scientific questions. As more experiments with the *Hint1* gene knockout mice are conducted, the contribution of HINT1 to normal physiology and to tumorigenesis will be elucidated.

2.2. HINT2

We identified human HINT2, a protein with 61% identity to HINT1, by screening a liver library (Fig. 1). Using real-time quantitative PCR and immunoblotting with a specific antibody, we discovered that HINT2 is predominantly expressed in the liver and the pancreas. Using immunohistochemistry, transfection with HINT2 fluorescent chimeric proteins and isolation of mitochondria, we determined that HINT2 is located in the mitochondria matrix [56].

Since HINT1 was first described to be a PKC inhibitor [5,57,58], we investigated whether HINT2 inhibited PKC. In a PKC activity assay, affinity-purified HINT2 did not inhibit the phosphorylation by PKC either of histone or of myelin basic protein, which discounts HINT2 as a PKC inhibitor. We tested whether HINT2 was a PKC substrate, since its sequence contains a potential PKC phosphorylation site (122TAK), albeit within an α-helix. In contrast to histone, HINT2 was not phosphorylated by a rat brain fraction enriched in PKCs. Neither overlay, nor co-immunoprecipitation revealed an interaction between HINT2 and protein kinase C isoforms [56].

Consistent with the fact that HIT proteins contain a nucleotide binding domain, Bieganowski et al. reported that substrates hydrolyzed at rates greater than 1 mmol·min^{-1}·μg^{-1} by Hint1 are adenosine 5′-monophosphoramide-linked compounds [2]. Hint1 also hydrolyzed AMP linked to a lysine side chain [59]. Given the high homology of HINT2 with HINT1, HINT2 was tested for an adenosine phosphoramidase activity. Using the model compound AMP-pNA, in which a paranitroaniline reporter is linked by a phosphoramidase linkage to AMP [2], we found that purified HINT2 had phosphoramidase activity, characterized by a k_{cat} of 0.0223 ± 0.0031 s^{-1} and a K_m of 128 ± 35 μM. In contrast, rabbit Hint1 activity was characterized by a k_{cat} of 0.00187 ± 0.00006 s^{-1} and a K_m of 134 ± 11 μM. Therefore, the k_{cat}/K_m of HINT2 is 10-fold larger than that of Hint1. When the middle histidine of the HIT motif of HINT2 was mutated to an alanine (HINT2–H149A), the adenosine phosphoramidase activity was lost.

The finding that HINT2 displays adenosine phosphoramidase activity and unlike HINT1, is located in the mitochondria, an organelle required for ATP synthesis and adenosine metabolism, broadens the search for

the physiological role of this HIT protein. Mitochondria regulate the cascade of apoptosis signals, which are triggered either extrinsically by the activation of death receptors or intrinsically by the disruption of intracellular homeostasis. Permeabilization of the mitochondrial membranes dissipates the potential of the inner membrane and allows the leakage of caspase-activating proteins into the cytoplasm, which in turn mediate apoptosis. In HepG2 cells overexpressing HINT2, the incubation with an antibody against Fas and actinomycin D induced greater changes in the mitochondrial membrane potential than in control HepG2 cell and the abundance of cleaved PARP and cleaved caspases 3, 7 and 9 was higher in the cells overexpressing HINT2. In the cell lines transfected with the hydrolase-negative HINT2–H149A and expressing less HINT2, exposure to anti-Fas antibody and actinomycin D resulted in less change in the mitochondrial membrane potential and less cleaved caspase 3.

In order to determine whether HINT2, like HINT1 displays tumor suppression properties, we assessed in vivo the influence of Hint2 on tumor growth. HepG2 cells were injected into the flank of SCID mice. HINT2-overexpressing HepG2 cells formed significantly smaller tumors than control HepG2 cells. This was associated with more cleaved caspase 9, uncleaved PARP and more apoptosis. This prompted us to analyze the expression of the HINT2 gene in several types of human tumors. Microarray analysis revealed that HINT2 expression was significantly reduced in hepatocellular carcinoma, but not in the surrounding cirrhotic tissue. In contrast, the expression of HINT1 and HINT3 mRNAs was not reduced in human hepatocellular carcinoma. HINT2 mRNA was less abundant in the subclass of HCC with a poor prognosis than in the subclass with better prognosis. Real-time quantitative PCR showed lower expression of HINT2 mRNA in breast carcinomas and in colon carcinomas than in normal tissue.

Since the most important regulatory steps of steroidogenesis occur in the mitochondria, we studied the role of HINT2 in the biosynthesis of pregnenolone in H295R adrenocortical cells [60]. When overexpressed, HINT2 effected no change in pregnenolone secretion elicited by angiotensin II or K^+, whereas when HINT2 was silenced with a specific siRNA, the steroidogenic response was reduced. Similarly, when HINT2 was overexpressed, the mitochondrion calcium signal did not change, whereas after silencing of HINT2, the duration of the mitochondrial calcium signal induced by angiotensin II was reduced. After HINT2 silencing, pregnenolone secretion was also reduced in response to activation of the cAMP pathway by forskolin and after exposure to 25-OH cholesterol, which bypasses the rate-limiting and Ca^{2+}-dependent step of mitochondrial import. These findings suggestthat HINT2 is required for an optimal steroidogenic response to both Ca^{2+}-dependent and Ca^{2+}-independent agonists but its role is not rate-limiting [60].

2.3. HINT3

A third human HINT gene is located at 6q22.33. HINT3 is predicted to encode a 182 amino acid protein with a 31 and 25 amino acid extension at the N and C terminus, respectively (Fig. 1) and is expressed in both cytosolic and nuclear compartments. HINT3 can assemble into multimeric oligomers and its substrate preference is acyl-adenylates over nucleoside phosphoramidates [61]. At present, no studies have investigated the expression or role of HINT3 in tumor formation or progression. We have generated Hint3$^{-/-}$ mice and are currently characterizing them.

2.4. Hint proteins in bacteria

The smallest of bacterial genomes (Mollicutes) [62] encode a Hint protein, therefore Hints may have been present at the cellular origin of life. In *E. coli*, the Hint orthologue echinT shares 50% identity with HINT1 and is essential for growth under high salt conditions [63]. EchinT forms stable interactions with a putative oxidoreductase, a formate dehydrogenate (bl501), the heat shock protein 70, the beta-subunit of DNA polymerase III, a membrane bound lytic murein transglycosylase D, ET-Tu elongation factor (tufA) and a putative synthetase (yjhH) [64].

3. Aprataxin

In 2001, linkage analysis showed that the APTX gene located on chromosome 9, was mutated in patients with ataxia–ocular apraxia, a neurological disorder with symptoms that resemble those of ataxia-telangiectasia, which is a syndrome characterized by an abnormal response to double-strand DNA breaks and genome instability [65,66]. APTX encodes Aprataxin, which is expressed in two splice forms and is located in the nucleus and nucleolus. In addition to a HIT motif, Aprataxin contains a C-terminal putative zinc finger domain, which can bind DNA [4,67] A third domain, the forkhead-associated domain, is present in the major splice form of Aprataxin, and this domain mediates the interaction with ligase cofactors, the x-ray cross-complementing proteins (XRCC). Although previously classified as a member of the HINT branch hydrolases [1], a more recent phylogenetic analysis indicates that Aprataxin warrants its place in a separate branch of the HIT superfamily [4].

Aprataxin possesses AMP-lysine and GMP-lysine hydrolase activity [68], and this encouraged the notion that reversal of nucleotidylylated protein modifications somehow underlies the biological actions of Aprataxin. Yeast two-hybrid system and co-immunoprecipitation studies found that Aprataxin interacts with the DNA repair proteins XRCC1 and poly(ADP-ribose) polymerase-1 (PARP-1) as well as with p53 [69]. Cells carrying a mutation in Aprataxin are more sensitive to the DNA topomerase I inhibitor, camptothecin, which induces DNA single-strand breaks [70] and a role for Aprataxin in DNA single-strand-break repair and in multiple DNA repair pathways has been documented recently [67,71]. At the molecular level, Aprataxin removes 5′AMP at DNA stand break sites [72]. Neurological disorders associated with Aprataxin mutations have been attributed to the accumulation of unrepaired DNA strand breaks [72]. In yeast genetic studies, deletion of the *S. cerevisiae* of the Hnt3 gene, homolog of Aprataxin, renders cells hypersensitive to DNA damaging agents when combined with a deletion in the flap endonuclease, Rad27, which results in deficiency in long-patch base excision repair [73]. The authors concluded that Hnt3/Aprataxin and flap endonucleases could process adenylated 5′ends of DNA in parallel. An association between coenzyme Q10 deficiency and a mutation in Aprataxin was identified in members of one family diagnosed with ataxia [74]. Microarray analysis of colorectal cancer cell lines showed a significant negative correlation between mRNA expression levels of Aprataxin and sensitivity to camptothecin-induced apoptosis [75]. Furthermore, a tissue microarray of tumor samples from colorectal cancer patients who were treated with the camptothecin analog, irinotecan, could distinguish a group with good response to therapy and who had longer progression free and overall survival when their tumor expressed less Aprataxin. The potential for Aprataxin expression levels to serve as a biomarker for the classification of tumors amenable to treatment awaits confirmation.

4. FHIT (Fragile HIT protein)

The FHIT gene is located within a fragile region of chromosome 3 of the human genome and is frequently altered in cancers and inactivated in cancer-derived cell lines [76]. The FHIT protein possesses a HIT motif and is a tumor suppressor. FHIT is absent or reduced in many types of human tumors including lung, esophagus, stomach, kidney and cervical carcinomas [77,78]. Deletions are observed in preneoplastic lesions of lung, cervix and breast [79]. Fhit$^{-/-}$ mice survive but spontaneously develop tumors more frequently than control mice [80]. Mice carrying one inactivated Fhit allele (Fhit$^{+/-}$) are highly susceptible to chemical induction of tumors [81].

Although first identified as a cytosolic protein, FHIT can be directed to the mitochondria upon interaction with the chaperones Hsp60 and Hsp10, where it interacts with ferredoxin reductase, a flavoprotein transactivated by p53 and responsible for transferring electrons from

NADPH [82]. The sorting of FHIT to the mitochondria is now recognized as essential to its tumor suppressor actions via apoptosis. The FHIT/ferrodoxin reductase complex generates reactive oxygen species and increases calcium uptake into the mitochondria to potentiate the effects of apoptotic agents [83].

The FHIT enzyme hydrolyzes dinucleoside polyphosphates (Ap_nA) although this hydrolysis appears to be independent from its tumor suppressor properties. Intracellular diadenosine 5′,5‴-P^1,P^n polyphosphates (Ap_nA, $n=3$–6) are signaling molecules whose concentrations are labile. Intracellular Ap_4A is increased in proliferating mammalian cells [84] and during liver regeneration [85,86]. Ap_3A is synthesized by tryptophanyl-tRNA-synthetase which, in contrast to other amino acyl tRNA synthetases, cannot produce Ap_4A. FHIT hydrolyzes Ap_3A to produce ADP and AMP [87], an enzymatic activity that was purified from rat liver in 1977 [88] but remained an orphan reaction until the discovery of FHIT. A mutated FHIT having lost Ap_3A hydrolase activity remains a nucleotide binding protein and a tumor suppressor [89]. An active signaling role was ascribed to the nucleotide-bound FHIT complex as a means of explaining the independence of the tumor suppressor and hydrolysis activities [89].

The tumor suppressor properties of FHIT are related to its pro-apoptotic effects. Human lung cancer cell lines overexpressing FHIT are less tumorigenic in nude mice and more frequently apoptotic [90,91]. Experimental evidence exists for protein interactions between FHIT and the MDM2 phosphoprotein, which interacts with p53 [92]. The FHIT-mediated inactivation of MDM2 blocked the association of MDM2 with p53, leading to the stabilization of p53 in human non-small cell lung carcinoma cells. Thus tumor cell growth was inhibited by coexpression of FHIT and p53 [92]. When FHIT expression was restored in FHIT-negative cell lines, caspase 8 was activated and apoptosis was induced [93]. These reports all indicate that FHIT can inhibit tumor growth in part via an apoptotic pathway. More recently, FHIT was found to be phosphorylated at Tyr 114 by Src kinase and to negatively regulate the central signaling molecule Akt [94] (Fig. 3).

Other molecular mechanisms must be considered to explain FHIT antitumoral activity. Weiske et al. overexpressed FHIT and β-catenin in HEK293 cells and showed that FHIT repressed transcriptional activity of β-catenin by direct interaction with its C-terminus and thereby regulated transcription of target genes such as cyclin D1 [95]. Therefore loss of FHIT would promote a deregulation of this pathway with consequences on proliferation, differentiation, and apoptosis. Woenckhaus et al. confirmed this hypothesis by immunohistochemical analysis of non-small cell lung cancer. They observed a loss of FHIT expression associated with poor prognosis and β-catenin expression [96]. Nakagawa and Akao over-expressed FHIT in the human colon cancer line, SW480, and reported a reduction in the phosphorylation of IκB-α [97]. The silencing of FHIT with siRNA promoted the phosphorylation and degradation of IκB-α resulting in the activation and nuclear translocation of NF-κB. In this experimental situation therefore, the anti-tumoral action of FHIT was attributed to the blocking NF-κB signaling pathway [97]. The role of FHIT may be different in metastatic tumors. The expression levels of various tumor associated proteins were determined by immunohistochemistry in tissue arrays of primary and metastatic gastric carcinoma. The level of FHIT as well as β-catenin and NF-κB was lower in gastric carcinoma metastasis than in primary tumors [98]. However, FHIT immunonegativity in the primary tumor was inversely correlated with advanced lymph node metastasis [98]. Other candidate FHIT-interacting proteins have been reported. FHIT co-immunoprecipitated with the ubiquitin-conjugating enzyme hUBC9 [99], suggesting a means of regulating the degradation of cell cyclins. The interaction with hUBC9 was subsequently shown to be dependent on Ap_3A binding to FHIT, although the hUBC9-FHIT binding complex was devoid of hydrolase activity [100]. The importance of the hUBC9-FHIT interaction awaits confirmation in an experimental tumor model.

5. Scavenger mRNA decapping enzyme, DcpS

The 3′→5′ mRNA exonuclease degradation pathway generates small, 5′-capped (m^7GpppN) mRNAs, which then become substrates

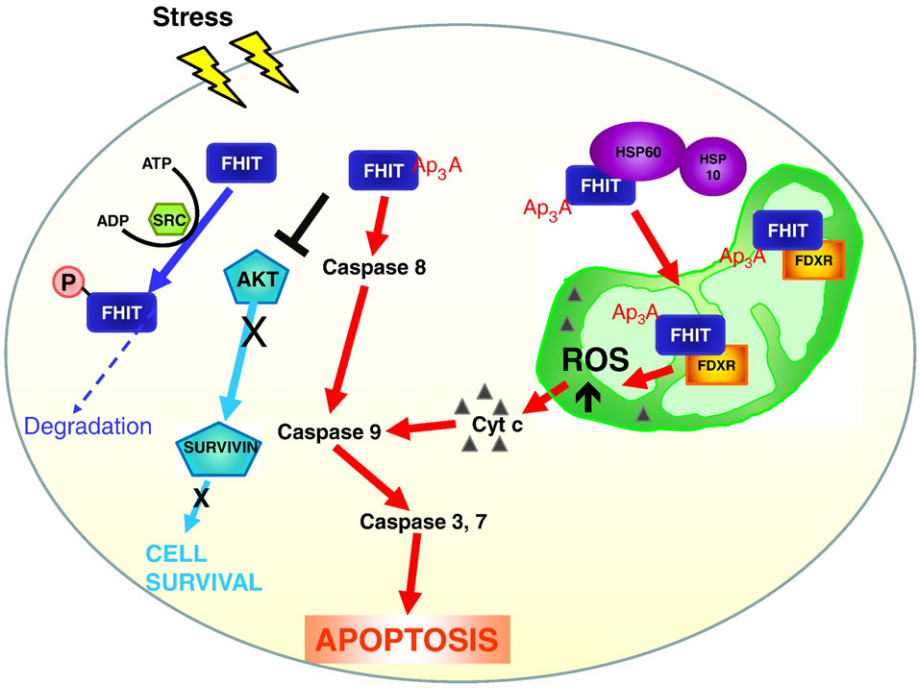

Fig. 3. The proposed mechanisms underlying the induction of apoptosis by the fragile HIT protein, FHIT. In response to cellular stress, the level of diadenosine polyphosphates increases. Ap_nA are substrates for FHIT and formation of Ap_nA·FHIT is required for apoptosis. FHIT can mediate apoptosis through the cytoplasmic pathway triggered by the activation of caspase 8. FHIT can also bind to the Hsp60/10 chaperone complex, which directs the bound protein to the mitochondria where it stabilizes ferrodoxin reductase (Fdxr). Reactive oxygen species (ROS) are produced, which engages the mitochondrial apoptotic pathway. FHIT also inactivates PI3K-Akt-Survivin signals. Src kinase phosphorylates FHIT at Tyr 114, which targets FHIT for proteosomal degradation.

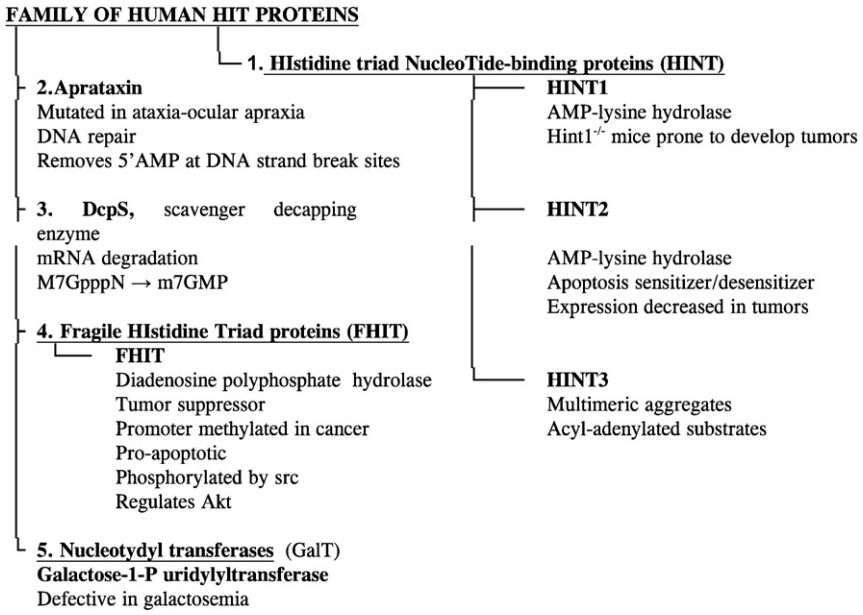

Fig. 4. Family of human hit proteins.

for further metabolism by the scavenger mRNA decapping enzyme (DcpS), a HIT protein expressed in the cytoplasm and nucleus [101,102]. The hydrolysis of m^7GpppG by DcpS yields m^7GMP and GDP. An extensive structure–activity relationship study indicated that the methylated base is an essential feature of the ligand and that cap analogues containing pyrimidine bases are not used as substrates [103]. The expression of DcpS is induced by the stress signals that result in the production of aborted mRNA transcripts. Delta-lactoferrin, a transcription factor involved in the regulation of cell cycle progression at the G1/S transition, activates DcpS transcription [104]. Information regarding the phenotype of animals in which the DcpS gene is disrupted is not yet available but by analogy with disruption of the yeast ortholog (dcs1Δ), DcpS regulates 5′ to 3′ exonucleolytic activity, which causes accumulation of short capped oligonucleotides as well as uncapped stable mRNAs [105]. As proposed by Bail and Kiledjian, it is logical that a reduction in the function of DcpS would cause an overall malfunction in mRNA splicing and decay, thereby modulating gene expression with consequences for tumorigenesis [106]. The most recent information concerning DcpS in human disease has identified DcpS as a molecular target of C5-quinazolines. Inhibition of DcpS by C5-quinazolines was associated with an increase in expression of the SMN2 gene, which can complement the defective levels of the survival motor neuron (SMN) protein that causes spinal muscular atrophy [107].

6. Galactose-1-phosphate uridylyltransferase (GalT)

The liver enzyme galactose-1-phosphate uridylyltransferase (GalT) catalyzes the transfer of UMP from UDP-glucose to galactose-1-phosphate to produce UDP-galactose and glucose-1-phosphate. GalT represents a separate branch of the HIT protein superfamily, which operates as a transferase rather than as a hydrolase. Its nucleotide transfer proceeds via a covalent intermediate formed between UMP and a His residue from its modified HIT motif, His-ϕ-His-ϕ-Gln-ϕ–ϕ. Mutations in the GalT gene result in galactosemia, a disease characterized by a failure to thrive, cataracts and mental retardation [1]. Unlike the other HIT proteins, GalT has a defined role in carbohydrate metabolism and is not implicated in tumorigenesis.

In conclusion, the HIT superfamily represents a diverse group of proteins, united by their structural motif but divergent in their tissue distribution, subcellular location, enzymatic activities and importance in tumorigenesis. As it now stands, the HINT and FHIT proteins have confirmed tumor suppressor activities, which could be mediated through regulation of transcription factors and pro-apoptotic mechanisms, and appears be independent of enzymatic activity. Within the HINT branch, we are most knowledgeable about the HINT1 protein, in terms of its expression in tumors and its purported mechanisms. Our study of, and appreciation for the significance of the HINT2 and HINT3 proteins lag far behind.

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