Control of dinucleoside polyphosphates by the FHIT-homologous HNT2 gene, adenine biosynthesis and heat shock in Saccharomyces cerevisiae

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Abstract

Background: The FHIT gene is lost early in the development of many tumors. Fhit possesses intrinsic ApppA hydrolase activity though ApppA cleavage is not required for tumor suppression. Because a mutant form of Fhit that is functional in tumor suppression and defective in catalysis binds ApppA well, it was hypothesized that Fhit-substrate complexes are the active, signaling form of Fhit. Which substrates are most important for Fhit signaling remain unknown.

Results: Here we demonstrate that dinucleoside polyphosphate levels increase 500-fold to hundreds of micromolar in strains devoid of the Saccharomyces cerevisiae homolog of Fhit, Hnt2. Accumulation of dinucleoside polyphosphates is reversed by re-expression of Hnt2 and is active site-dependent. Dinucleoside polyphosphate levels depend on an intact adenine biosynthetic pathway and time in liquid culture, and are induced by heat shock to greater than 0.1 millimolar even in Hnt2+ cells.

Conclusions: The data indicate that Hnt2 hydrolyzes both ApppN and AppppN in vivo and that, in heat-shocked, adenine prototrophic yeast strains, dinucleoside polyphosphates accumulate to levels in which they may saturate Hnt2.

Background

The human FHIT gene, located at the chromosome 3 fragile site FRA3B, is inactivated early in the development of many tumors [1]. Murine Fhit is also located at a fragile site [2,3] and mice heterozygous for disruption of Fhit, given low intragastric doses of the mutagen N-nitrosomethylbenzylamine, develop stomach and sebaceous tumors [4] that can be prevented by viral Fhit expression [5]. Fhit, a dimer of 147 amino acid subunits, is a member of the histidine triad (HIT) superfamily of nucleotide hydrolases and transferases [6,7]. Members of the Hint branch of the HIT superfamily are found in all forms of life [8]. The S. cerevisiae Hint homolog, Hnt1, and rabbit Hint possess adenosine monophosphoramidase activity that functions in yeast to positively regulate function of Kin28, Ccl1 and Tfb3, which constitute the kinase compo-
nent of general transcription factor TFIIH [9]. A new Hint related protein, Apratx, is mutated in individuals with ataxia with oculomotor apraxia [10,11] and has a yeast homolog termed Hnt3 [9]. Members of the Hnt branch of the HIT superfamily have been found in fungi [12,13], animals [2,14,15] and plants [7] and hydrolyze diadenosine tetraphosphate, diadenosine triphosphate and other 5'-5'-dinitoside polyphosphates. The middle histidine of the histidine triad (His96 in human Fhit), which is critical for hydrolysis of ApppA by Fhit [14,16], is not necessary for tumor suppression [17,18]. Nonetheless, wild-type and His96Asn forms of Fhit are saturated by ApppA in the low micromolar range and form stable complexes with non-hydrolyzable ApppA in which two ApppA analogs are bound per Fhit dimer and all phosphates cluster on one surface of the protein [16]. These observations suggested that Fhit-substrate complexes may be the active, signaling form of Fhit and that the function of the catalytically essential histidine may be to terminate the lifetime of signaling complexes [16].

Given that neither transcriptional nor post-transcriptional regulation has been reported for Fhit protein, the level of biological activity of Fhit may be controlled by levels of Fhit substrates, inhibitors, and proteins that interact with Fhit-nucleotide complexes. Fhit proteins from humans [19] and worms [15] bind ApppA and AppppA with $K_m$ values of 2 to 3 $\mu$M. Human Fhit [14] and the S. cerevisiae Fhit homolog [13], which was called Aph1 but is here termed Hnt2 under nomenclature approved by the Saccharomyces Genome Database, cleave ApppA more readily while Aph1, the S. pombe homolog, cleaves ApppA more readily [20]. Consistent with the ApppA hydrolase activity of purified Fhit protein, most cancer cell lines that are Fhit negative at the protein level have higher levels of ApppA than cell lines that are Fhit positive [21]. Nonetheless, the actual concentrations of dinucleoside polyphosphates were submicromolar in every cell culture sample [21] and thus, under the reported culture conditions, the measured dinucleoside polyphosphates would not be expected to occupy the Fhit active site substantially [19]. Dinucleoside polyphosphate levels were measured in adenine-requiring S. cerevisiae strains before or after disruption of the Fhit-homologous HNT2 gene [13] and in adenine-requiring S. pombe strains as a function of disruption and overexpression of the Fhit-homologous aph1 gene [22]. Recently, it was observed that diadenosine polyphosphates undergo a divalent cation-dependent conformational change that might mediate their biosynthesis, catabolism or signaling properties [23].

Here we discover a requirement of adenine biosynthesis for high-level dinucleoside polyphosphate accumulation in the absence of the Fhit homolog in S. cerevisiae. By constructing active site mutants of Hnt2 that were expressed in yeast, we demonstrate that ApppN and, to a lesser degree, AppppN levels are controlled by the Hnt2 active site. An added benefit of these constructions is the availability of yeast strains that possess high levels of dinucleoside polyphosphates and at the same time express a mutant Fhit-homologous protein, because these are conditions which have been postulated to constitute the signaling form of Fhit [16]. Finally, using controlled genotypes we revisited conditions that lead to increased accumulation of dinucleoside polyphosphates [24–33]. Recognizing that hnt2 deletion is a pathological condition, we were particular interested in identifying conditions that lead to accumulation of such compounds in cells that contain a functional HNT2 gene, rather than simply identifying conditions that produce diadenosine polyphosphate accumulation in the absence of Hnt2. While cells without a functional HNT2 gene accumulate dinucleoside polyphosphates in excess of 10 $\mu$M in a variety of non-stressed and stressed conditions, 46°C heat shock was the only condition that produced dinucleoside polyphosphate accumulation in excess of 10 $\mu$M in cells containing a functional HNT2 gene. These conditions did not render the cells conditionally null for Hnt2 because cells expressing HNT2 continued to limit dinucleoside polyphosphate accumulation during hours of heat shock, though at levels of ~0.1 mM.

Recently, discovery that the Hint-homologous HNT1 gene is required for high temperature growth on galactose and observations that allelic of cak1, kin28, ccl1 and ffb3 are hypersensitive to loss of Hnt1 enzyme activity provided evidence that Hnt1 enzyme activity positively regulates Kin28 function, particularly on galactose media [9]. Though phenotypic consequences of hnt2 mutations have yet to be discovered, our observations suggest that synthetic lethal interactions with hnt2 mutations are likely to be found in adenine prototrophic strains undergoing heat shock.

Results and discussion

Disruption of HNT2 and tetrad analysis of dinucleoside polyphosphate levels

An earlier report demonstrated that disruption of HNT2 was tolerated by haploid yeast strains without an effect on growth and that ApppN and AppppN accumulate 30 and 3-fold, respectively on account of the hnt2 deletion [13]. Because those data were obtained by random spore analysis, we considered it important to test whether elevated dinucleoside polyphosphate levels co-segregate with hnt2 disruption in all tetrads examined and whether any other commonly used genetic markers affect dinucleoside polyphosphate levels. Diploid strain BY71 (Table 1), created to be heterozygous for MAT, ADE2, HIS3, LEU2, LYS2, MET15, TRP1, URA3 and HNT2, was allowed to sporulate and was then dissected. As shown in Figure 1,
tetrads produced four viable colonies, two of which were large and white, and two of which were smaller and pink on YPD medium, which scored as ade- on SDC -ade medium. Markers segregated 2:2 in nearly all cases and possession of a 1976 bp PCR fragment using primers 4726 and 4722 always correlated with geneticin-resistance while possession of a 1200 bp product correlated with geneticin-sensitivity, as expected for segregants containing a disrupted HNT2 gene.

Haploid segregants from three complete tetrads were cultured for 24, 48 and 72 hours in SDC medium, lysed, and levels of ApppN and AppppN were determined. As shown in Table 2, strains containing an intact HNT2 gene were never observed to have calculated intracellular ApppN levels above 3 μM and typically were found to have ApppN levels below 1 μM. Strains disrupted for hnt2 had ApppN levels of approximately 6 to 43 μM after one day of culture, rising to approximately 30 to 300 μM and 50 to 350 μM after two and three days of culture, respectively. The ade2 mutation was partially epistatic to the partial disruption of hnt2 on ApppN accumulation. hnt2ΔADE2 strains containing ade2 mutations were several fold lower in ApppN accumulation than hnt2ΔADE2 isolates. Thus, deletion of hnt2 afforded a 48-fold increase in ApppN in ade2 mutants, consistent with an earlier report of a 31-fold effect [13], but a 211-fold increase in ADE2 strains.

Earlier, hnt2 deletion was reported to increase AppppN levels only 2.5-fold but the study was performed in ade2 mutants [13]. Consistent with that report, the three hnt2 ade2 strains showed only a 2-fold higher AppppN level than the three HNT2 ade2 strains, when nucleotide levels were averaged across the three time points. In contrast, hnt2ΔADE2 strains achieved a 3.7-fold higher level of AppppN than HNT2 ADE2 strains. Larger increases in AppppN concentrations have been seen with disruption of Apa1 and Apa2, the diadenosine tetraphosphate phosphorolases in S. cerevisiae [34,35], indicating that they have a more significant role in controlling AppppN levels than does Hnt2. In the case of disruption of the Fhit and Hnt2-homologous aph1 gene in S. pombe, which encodes an enzyme relatively specific for a AppppA [20], a 290-fold increase in AppppA concentration was observed [22]. Our data indicate that Hnt2 hydrolyzes ApppN and AppppN in vivo in budding yeast and that an intact adenine biosynthetic pathway is required for high-level synthesis and accumulation of adenylylated dinucleoside polyphosphates.

**Hnt2 active site-dependence of dinucleoside polyphosphate accumulation**

Catalytic activity of the Fhit ApppA hydrolase depends on His96 [14,16]. To test whether the active site of Hnt2 is necessary to control dinucleoside polyphosphate accumulation in vivo, wild-type and mutant alleles of HNT2 that differ at His109, corresponding to human Fhit His96,
were expressed from the HNT2 promoter on plasmids (Table 3) in hnt2ADE2 strain BY71-6c. As shown in Table 4, reintroduction of wild-type HNT2 produced a 40 to 125-fold reduction in intracellular concentrations of ApppN and a two to seven-fold reduction in levels of AppppN. This reduction was active-site dependent: adding back multicopy HNT2 with the nucleophilic histidine replaced by alanine or aspartate reduced dinucleoside polyphosphate levels less than two-fold.

**Dinucleoside polyphosphate levels may not be limited by the levels of lysyl tRNA synthetase**

AppppA is induced by heat shock in bacteria [36] and the induction of AppppA was thought to be a function of the heat-shock inducible LysU lysyl tRNA synthetase. However, deletion of lysU had no effect on heat-shock inducible AppppA accumulation [37]. The KRS1 gene [38] encoding cytosolic lysyl tRNA synthetase was cloned into multicopy plasmid pRS423 [39] to generate plasmid pM1. Strains BY71-16d (ADE2 HNT2) and BY71-6c (ADE2 hnt2) were transformed with pM1 and the pRS423 control plasmid, and cultures were harvested at 24, 48 and 72 hours. Determination of ApppN and AppppN concentrations revealed that ApppN levels are substantially higher in hnt2 mutants than in isogenic wild-types at all time points and that plasmids conferring multiple copies of KRS1 did not increase ApppN or AppppN levels at any culture time point (Table 5). To address whether plasmid pM1 indeed increased lysyl tRNA synthetase activity, lysates from pRS423 and pM1-transformed BY71-6c were assayed for incorporation of 3H lysine into yeast tRNA. As shown in Figure 2, tRNA-dependent lysine incorporation was increased 2.1-fold by expression of KRS1 from a multicopy plasmid.

**Heat shock is the most effective stress for elevation of dinucleoside polyphosphates**

In the wild and in the laboratory, yeast are exposed to stresses such as hypo-osmotic or hyperosmotic conditions, toxic cations, heat shock and cell-cycle disruptive reagents. To test whether such conditions induce dinucleoside polyphosphates in hnt2- or Hnt2+ cells, we incubated ADE2 hnt2 and ADE2 HNT2 cells in water, 1 M sorbitol, 2 mM CdCl₂, 46 °C heat shock, 10 mM caffeine, or in rich media for two hours and determined dinucleoside polyphosphate levels. Additionally, to test whether moderate overexpression of the lysyl tRNA synthetase gene affected accumulation, we compared control transformants to multicopy KRS1 transformants of the two strains. As shown in Table 6, the hnt2 samples had substantially higher ApppN levels than HNT2 samples under all conditions. Among the hnt2 samples, only heat shocked samples showed evidence of AppppN levels higher than the levels in nonstressed hnt2 cells. Similarly, among the HNT2 samples, the heat shocked samples showed increased ApppN levels compared with control-treated cells while CdCl₂ and other treated samples showed no significant changes. KRS1 on a multicopy plasmid showed no significant alteration of ApppN levels in any sample. As with other experiments, AppppN levels were lower than ApppN levels in all cases. Heat shock was the best inducer.
of AppppN. Hypotonic, hypertonic and caffeine treated media produced no increase in AppppN (not shown).

To further investigate the kinetics of heat shock and cadmium-induction of ApppN and AppppN levels, we transformed hnt2∆ADE2 strain BY71-6c with multicopy plasmids containing no HNT2 gene, the wild-type HNT2 gene, or the HNT2-His109Ala or HNT2-His109Asp alleles of HNT2. Cultures were exposed to either 2 mM CdCl2 or 46°C heat shock and intracellular concentrations of ApppN and AppppN were determined at 30-minute time-points.

As shown in Figure 3, ApppN and AppppN levels are higher in hnt2 strains than in cells with a functional HNT2 gene, and were not significantly elevated by 2 mM CdCl2. However, when cells were heat shocked, as shown in Figure 4, ApppN and AppppN levels increased substantially in cultures with every HNT2 genotype (absence, presence or active site mutation). Increases in dinucleoside polyphosphates in Hnt2+ cultures cannot be attributed to thermal inactivation of Hnt2 because the presence of HNT2 plasmids continues to reduce incremental increases in dinucleoside polyphosphates even in the fourth hour of the heat shock. Furthermore, the active-site mutant alleles of HNT2, HNT2-His109Ala and HNT2-His109Asp, provided on plasmids pB32 and pB86, demonstrated intermediate abilities to control dinucleoside polyphosphate levels, suggesting that elevated temperature reduces the catalytic defects of these mutant enzymes. The high levels of AppppN (~100 µM) and AppppN (~10 µM) and the fact that Hnt2-containing samples continue to reduce the rate of increase in dinucleoside polyphosphates without reducing their concentrations demonstrate that heat shock induces dinucleoside polyphosphate synthesis and that Hnt2 is saturated under such conditions. In Xenopus oocytes, however, some work has suggested that heat shock-dependent accumulation of AppppN is largely due to inactivation of degradative enzymes [30].

**Conclusions**

It had been reported that Hnt2 controls ApppN levels in vivo, with a minor effect on AppppN [13]. Here we show that the ade2 mutation present in earlier experiments prevents accumulation of ApppN and AppppN and reduced the magnitude of the Hnt2 effect. In ADE2 strains examined herein, deletion of HNT2 increased levels of ApppN and AppppN by factors of approximately 200 and 4, respectively. Mutagenesis [14], X-ray crystallography [16,40], and stereochemical analysis [41] indicate that
His96 is the nucleophile that attacks the $\alpha$-phosphate of Fhit substrates. Our analysis shows that the corresponding residue, His109 of Hnt2, is required for hydrolysis of ApppN and AppppN substrates in vivo.

Other than effects on the concentrations of intracellular nucleotides, neither deletion of HNT2 nor mutation of His109 of Hnt2, is required for hydrolysis of ApppN and AppppN substrates in vivo.

Table 4: Intracellular concentration ($\mu$M) of dinucleoside polyphosphates controlled by the Hnt2 active site

| Plasmid in strain BY71-6c | Culture time | 24 hr | ApppN | AppppN | Genotype |
|---------------------------|--------------|-------|-------|--------|----------|
| None                      |              |       |       |        |          |
| pRS423                    |              | 20.70 | 0.16  | 129.00 | hnt2∆    |
| pB05                      |              | 23.80 | 0.21  | 140.00 | hnt2∆    |
| pB32                      |              | 18.70 | 0.17  | 201.00 | HNT2-His109Ala |
| pB86                      |              | 13.40 | 0.16  | 80.40  | HNT2-His109Asp |

Table 5: Intracellular concentration ($\mu$M) of dinucleoside polyphosphates as a function of culture time, HNT2 genotype, and presence of multicopy lysyl-tRNA synthetase gene

| Nucleotide | Culture Time | 24 hr | ApppN | 48 hr | AppppN | 72 hr | 24 hr | 48 hr | 72 hr |
|------------|--------------|-------|-------|-------|--------|-------|-------|-------|-------|
| HNT2       |              | 0.52  | 1.60  | 0.22  | 0.29   | 0.19  | 0.02 |
| HNT2 YEpKRS1 |            | 1.28  | 1.29  | 0.20  | 0.34   | 0.22  | 0.01 |
| hnt2∆      |              | 6.25  | 14.10 | 3.54  | 0.35   | 0.26  | 0.02 |
| hnt2∆ YEpKRS1 |          | 5.83  | 15.50 | 4.20  | 0.40   | 0.30  | 0.01 |

Materials and methods

General molecular biology

Yeast media and procedures were as described [43,44]. S. cerevisiae transformations were carried out by the lithium acetate method [45]. E. coli strain XL-1 Blue was used for bacterial cloning and plasmid amplification. Bacterial media and molecular biology techniques were as described [44].
Disruption of \textit{hnt2}

A 1570 bp DNA fragment containing an \textit{hnt2}:\textit{kanMX2} disruption cassette was generated as described [46]. Primers 4716 (5’GAAGCTCCATTGATCTATCTTGGGCTCAGAATGATCTTAAGCAAAACAAAGCTTCGTACGCTGCAG) and 4717 (5’CGTAAGTATGAATCTATTATTTATTGAACTATAGTTATTAAACCAGGGCCACTAGTGGATCTGA) were used to amplify the yeast expressible geneticin-resistance gene from \textit{pFA6a-kanMX2} [46] with 50 bp DNA ends corresponding to sequences upstream and downstream of \textit{HNT2}. The resulting fragment was ligated to \textit{Hnt2} disrupted to generate \textit{hnt2}:\textit{kanMX2} product in place of the wild-type 1200 bp \textit{HNT2} product was named strain BY16. Strain BY16 was crossed with strain BY4717 [47] to generate diploid strain BY71. Strain BY71 was allowed to sporulate and subjected to tetrad dissection to generate haploid strains BY71-1a through BY71-16d. Genotypes of all yeast strains are provided in Table 1.

\textbf{Plasmid constructions}

The \textit{HNT2} gene was amplified from genomic DNA of yeast strain SEY6210 [48] with primers PB1 (5’GCAGCGCTTGGGAT) that spanned a \textit{BamHI} site upstream of the promoter and PB2 (5’GAGTCTCCTCGAGGAAAG) that spanned a \textit{XhoI} site downstream of the terminator. The 1316 bp \textit{BamHI-XhoI} fragment containing \textit{HNT2} was ligated to \textit{BamHI} and \textit{XhoI}-cleaved plasmid pRS423 [39] to generate plasmid pB05. Plasmids pB32 and pB86, carrying \textit{H109A} and \textit{H109D} alleles of \textit{HNT2}, were constructed by site-directed mutagenesis [49] of plasmid pB05 using primers PB3 (5’ATAATGTGTGTAGCCAAGTGAGGGTGAC) and PB4 (5’TACGATCTCGAGGAAAG). The \textit{S. cerevisiae} gene encoding lysyl tRNA synthetase (\textit{KRS1}) was amplified as a 2.9 kbp genomic fragment from strain SEY6210 using primers MR20 (5’CGAGCTCGGTTGGATCTTTAAAATGACTATAAC) and MR21 (5’TCCCCGGGAGCTCCTTGAGCTGACGAG). This product, cloned into the \textit{SmaI} restriction site of plasmid pRS423, generated plasmid pM1 in which \textit{KRS1} is oriented anti to \textit{HIS3}. Plasmids are summarized in Table 3.

\textbf{Measurement of dinucleoside polyphosphate levels}

Twelve haploid segregants, pregrown in liquid SDC medium, were inoculated into 250 ml of SDC at starting density of 10^4 cells per ml. At 24, 48 and 72 hours of growth, 50 ml of cells were harvested, cells counted microscopically, lysed, and levels of AppppN and ApppN were determined as described [13]. Intracellular concentrations of AppppN and ApppN were calculated using 7 × 10^{-14} l as the volume of a haploid cell [50]. Cultures of BY71-6c were transformed with plasmids pRS423, pB05, pB32 and pB86 and transformants were selected on SDC-his media. Intracellular concentrations of ApppN and AppppN were determined for transformants from cultures in SDC-his
media as above. To determine whether a multicopy lysyl tRNA synthetase plasmid affected accumulation of dinucleoside polyphosphates, we transformed \( \textit{HNT2 ADE2} \) strain BY71-16d and \( \textit{hnt2} \Delta \textit{ADE2} \) strain BY71-6c with control plasmid pRS423 and with plasmid pM1. Transformants were grown for 24, 48 and 72 hours in SDC-his media and intracellular dinucleoside polyphosphate concentrations were determined as above. To survey dinucleoside polyphosphate induction as a function of potential stress conditions, strain BY71-6c was transformed with either pRS423 or pM1 (effectively \( \textit{hnt2} \Delta \textit{A} \) and \( \textit{hnt2} \Delta \textit{YEpKRS1} \), respectively) and strain BY71-16d was transformed with the same plasmids (effectively \( \textit{HNT2} \Delta \textit{A} \) and \( \textit{HNT2} \Delta \textit{YEpKRS1} \), respectively). After 48 hours of culture, cells were pelleted and resuspended in either YPD media, water, SDC-his media, or the same media supplemented with 2 mM CdCl\(_2\), 10 mM caffeine or 1 M sorbitol. The SDC-his sample was incubated for 2 hours at 46°C while

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**Figure 3**

\textbf{Cadmium is a poor inducer of dinucleoside polyphosphates} Calculated intracellular concentrations of ApppN and AppppN of transformants of strain BY71-6c as a function of time in 2 mM CdCl\(_2\).
other samples were incubated for 2 hours at room temper-
剖ure prior to extraction for determination of dinucleoside
polyphosphate concentrations. To determine the time
course of ApppN and AppppN levels as a function of
stresses, we used strains BY71-16d and BY71-6c trans-
formed with pRS423, pB05, pB32, or pB86. Transform-
ants were cultured for 48 hours, treated with 2 mM CdCl₂
or 46°C heat shock, and then harvested for nucleotide
quantitation at 30 minute intervals. Experiments present-
ed in Tables 2, 4, 5 and 6 were performed two, three, five
and three times, respectively. Experiments presented in
Figures 3 and 4 were performed four times each. Because
diadenosine polyphosphate levels vary with time in cul-
ture, generating a higher or lower range of values in inde-
pendently conducted experiments, we did not average
values obtained in separate experiments. For the data pre-
sented in Table 6, triplicate cultures were prepared and the

Figure 4
Heat shock induces dinucleoside polyphosphates Calculated intracellular concentrations of ApppN and AppppN of
transformants of strain BY71-6c as a function of time at 46°C.
AppnP levels for identically treated samples are provided as averages ± standard deviations.

**Lysyl tRNA synthetase activity assay**

Strain BY71-6c was transformed with plasmids pRS423 and pM1 (multicopy KRS1) and cultures were grown as for measurement of dinucleoside polyphosphate levels. Lysates were prepared by glass bead disruption in 50 mM Tris Cl pH 7.5, 1 mM DTT, 40% glycerol. Incorporation of tritiated lysine into tRNA was measured by modification of the protocol of Hou [51]. Reactions contained 10 micrograms of total protein in 20 mM KCl, 10 mM MgCl₂, 4 mM dithiothreitol, 2 mM ATP, 50 mM Na HEPES pH 7.5, 20 µM lysine (10 µCi ³H lysine), and were performed with or without 15 µg yeast tRNA (Sigma) in a total volume of 60 µl. At one, two, three, five and twelve minute time points, 10 µl aliquots were spotted on filter paper and placed in 5% wt/vol trichloroacetic acid, washed in 5% 2. Pekarsky Y, Campiglio M, Siprashvili Z, Druck T, Sedkov Y, Tillib S, Glover TW, Hoge AW, Miller DE, Ascara-Wilke JE, Adam AN, Dierick HA, Beer DG: The murine Fhit gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. Proc Natl Acad Sci USA 1998, 95:5484-9

**References**

1. Ohta M, Inoue H, Corticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Barnes LD: The gene mutated in Muir-Torre-like syndrome encodes diadenosine tetraphosphate (Ap4A) asymmetrical hydrolase: sequence similarity with the histidine triad (HIT) protein family. Biochem J 1995, 312:925-932
2. Moreira MC, Barbot C, Tachi N, Kozuka N, Uchida E, Gibson T, Mendonça P, Costa M, Barros J, Yanagisawa T, et al: The gem mutated in ataxia-oculopathy apraxia 1 encodes the new HIT/Zn-finger protein apratxin. Nat Genet 2001, 29:189-93
3. Huang Y, Garrison PN, Barnes LD: Cloning of the Schizosaccharomyces pombe gene encoding diadenosine 5',5'P₄-tetraphosphate (Ap₄A) by physical and biochemical characterization of Fhit-substrate complexes as the active signaling form of Fhit. Proc Natl Acad Sci USA 1998, 95:5484-9
4. Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganowski P, Pekarsky Y, Croce CM, Brenner C: Crystal structure of the worm NfHit Rosetta Stone protein reveals a Nt tetramer binding two Fhit dimers.Curr Biol 2000, 10:907-17
5. Draganescu A, Hodawadekar SC, Ge KR, Brenner C: Fhit-nucleotide specificity probed with novel fluorescent and fluorogenic substrates. J Biol Chem 2000, 275:4555-4560
6. Robinson AK, de la Pena CE, Barnes LD: Isothermal titration calorimetry Reveals a Zinc Ion as an Atomic Switch in the Diadeno-8

**Authors' notes**

Tetrads shown in figure 1 were dissected and PCR analyzed to show the procedure followed to obtain strains. They do not correspond to tetrads in table 1.

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24. Lee PC, Bochner BR, Ames BN: AppppA, heat-shock stress, and cell oxidation. Proc Natl Acad Sci USA 1983, 80:7496-500
25. Bochner BR, Lee PC, Wilson SW, Cutler CW, Ames BN: AppppA and related adenyllylated nucleotides are synthesized as a consequence of oxidative stress. Cell 1984, 37:225-32
26. Brevet A, Plateau P, Best-Belpomme M, Blanquet S: Variation of Ap4A and other dinucleoside polyphosphates in stressed Drosophila cells. J Biol Chem 1985, 260:15566-70
27. Baker JC, Jacobson MK: Alteration of adenyl dinucleotide metabolism by environmental stress. Proc Natl Acad Sci USA 1986, 83:2350-2
28. Baltzinger M, Ebel JP, Remy P: Accumulation of dinucleoside polyphosphates in Saccharomyces cerevisiae under stress conditions. High levels are associated with cell death. Biochimie 1986, 68:1231-6
29. Garrison PN, Mathis SA, Barnes LD: In vivo levels of diadenosine tetraphosphate and adenosine tetraphospho-guanosine in Physarum polycephalum during the cell cycle and oxidative stress. Mol Cell Biol 1986, 6:1179-86
30. Guedon GF, Gilson GJ, Ebel JP, Befort NM, Remy PM: Lack of correlation between extensive accumulation of bisnucleoside polyphosphates and the heat-shock response in eukaryotic cells. J Biol Chem 1986, 261:6455-69
31. Coste H, Brevet A, Plateau P, Blanquet S: Non-adenyllylated bis(5'-nucleosidyl) tetraphosphates occur in Saccharomyces cerevisiae and in Escherichia coli and accumulate upon temperature shift or exposure to cadmium. J Biol Chem 1987, 262:12096-103
32. Garrison PN, Mathis SA, Barnes LD: Changes in diadenosine tetraphosphate levels in Physarum polycephalum with different oxygen concentrations. J Bacteriol 1989, 171:1506-12
33. Johnston DJ, Hart CA, McLennan AG: Variation in intracellular P1P4-bis(5'-adenosyl) tetraphosphate (Ap4A) in virus-infected cells. Biochem J 1990, 268:791-3
34. Plateau P, Fromant M, Schmitter JM, Blanquet S: Catabolism of bis(5'-nucleosidyl) tetraphosphates in Saccharomyces cerevisiae. J Bacteriol 1990, 172:6893-9
35. Avila DM, Robinson AK, Kaushal V, Barnes LD: A paradoxical increase of a metabolite upon increased expression of its catalytic enzyme: the case of diadenosine tetraphosphate (Ap4A) and Ap4A phosphorylase I in Saccharomyces cerevisiae. J Bacteriol 1991, 173:7875-80
36. Kitzler JW, Farr SB, Ames BN: Intracellular functions of ApnN-prokaryotes. In: Ap4A and Other Dinucleoside Polyphosphates (Edited by: AG McLennan) Boca Raton, FL: CRC Press 1992
37. Brevet A, Chen J, Levque F, Blanquet S, Plateau P: Comparison of the Enzymatic Properties of the Two Escherichia coli Lysyl-tRNA Synthetase Species. J Biol Chem 1995, 270:14439-14444
38. Miranda M, Waller JP: The yeast lysyl-tRNA synthetase gene. Evidence for general amino acid control of its expression and domain structure of the encoded protein. J Biol Chem 1988, 263:18443-51
39. Christanson TW, Sikorski RS, Dante M, Shero JH, Hieter P: Multi-functional yeast high-copy-number shuttle vectors. Gene 1992, 110:119-122
40. Lima CD, Klein MG, Hendrickson WA: Structure-based analysis of catalysis and substrate definition in the HIT protein family. Science 1997, 278:286-90
41. Abend A, Garrison PN, Barnes LD, Frey PA: Stereochemical retention of configuration in the action of Fhit on phospho-chiral substrates. Biochemistry 1999, 38:3668-3676
42. McLennan AG: Dinucleoside polyphosphates-friend or foe? Pharmacol Ther 2000, 87:73-89
43. Kaiser C, Michaelis S, Mitchell A: Methods in Yeast Genetics. Plainview, NY: Cold Spring Harbor Laboratory Press; 1994
44. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: Short Protocols in Molecular Biology. New York: Wiley 1995
45. Gietz RD, Schiesth RH, Willems AR, Woods RA: Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 1995, 11:355-60
46. Wach A, Brachat A, Pohlmann R, Philippso: New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast 1994, 10:1793-808
47. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD: Designer deletion strains derived from Saccharomyces cer-