BMC Molecular Biology

Research article

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

Marta Rubio-Texeira[1,2], James M Varnum[1], Pawel Bieganowski[1] and Charles Brenner*[1]

Address: [1]Structural Biology & Bioinformatics Program, Kimmel Cancer Center, Philadelphia, Pennsylvania, USA and [2]Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA

E-mail: Marta Rubio-Texeira - mrubiotx@mit.edu; James M Varnum - varnum@dada.jci.tju.edu; Pawel Bieganowski - pawel@dada.jci.tju.edu; Charles Brenner* - brenner@dada.jci.tju.edu

*Corresponding author

Published: 20 May 2002

BMC Molecular Biology 2002, **3**:7

Received: 31 January 2002
Accepted: 20 May 2002

This article is available from: http://www.biomedcentral.com/1471-2199/3/7

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, Aprataxin, is mutated in individuals with ataxia with oculomotor apraxia [10,11] and has a yeast homolog termed Hnt3 [9]. Members of the Fhit 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"'-dinucleoside 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 μM. Human Fhit [14] and the *S. cerevisiae* Fhit homolog [13], which was called Aph1 but is here termed Hnt2 under nomenclature aproved by the *Saccharomyces* Genome Database, cleave ApppA more readily while Aph1, the *S. pombe* homolog, cleaves AppppA 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 μM 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 μM 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 alleles of *cak1*, *kin28*, *ccl1* and *tfb3*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,

Table 1: *S. cerevisiae* strains in this study

Name	Genotype	Background and source
SEY6210	*MATα his3Δ200 leu2-3,112 lys 2-81 suc2-Δ9 trp1-Δ901 ura3-52*	S288C, [48]
BY4717	*MAT***a** *ade2Δ::hisG*	S288C, [47]
BY4727	*MATα his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0*	S288C, [47]
BY16	*MATα his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hnt2Δ::kanMX2*	BY4727, this work
BY71	*MAT***a**/MATα *ADE2/ade2Δ::hisG HIS3/his3Δ200 LEU2/leu2Δ0 LYS2/lys2Δ0 MET/met15Δ0 TRP1/trp1Δ63 URA3/ura3Δ0 HNT2/hnt2Δ::kanMX2*	BY4717 × BY16, this work
BY71-1a	*MATα his3Δ200 leu2Δ0 lys2Δ0 hnt2Δ::kanMX2*	BY71 segregant, this work
BY71-1b	*MATα ade2Δ::hisG his3Δ200 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hnt2Δ::kanMX2*	BY71 segregant, this work
BY71-1c	*MAT***a** *ade2Δ::hisG leu2Δ0 met15Δ0 ura3Δ0*	BY71 segregant, this work
BY71-1d	*MAT***a** *trp1Δ63*	BY71 segregant, this work
BY71-2a	*MATα ade2Δ::hisG leu2Δ0 met15Δ0 hnt2Δ::kanMX2*	BY71 segregant, this work
BY71-2b	*MATα his3Δ200 leu2Δ0 lys2Δ0 met15D0 ura3Δ0 hnt2Δ::kanMX2*	BY71 segregant, this work
BY71-2c	*MAT***a** *ade2Δ::hisG his3Δ200 lys2Δ0 trp1Δ63 ura3Δ0*	BY71 segregant, this work
BY71-2d	*MAT***a** *trp1Δ63*	BY71 segregant, this work
BY71-4a	*MAT***a** *lys2Δ0 ura3Δ0 hnt2Δ::kanMX2*	BY71 segregant, this work
BY71-4b	*MATα his3Δ200 met15Δ0 ura3Δ0*	BY71 segregant, this work
BY71-4c	*MAT***a** *ade2Δ::hisG his3Δ200 leu2Δ0 met15Δ0 trp1Δ63*	BY71 segregant, this work
BY71-4d	*MATα ade2Δ::hisG leu2Δ0 lys2Δ0 trp1Δ63 hnt2Δ::kanMX2*	BY71 segregant, this work
BY71-6c	*MAT***a** *his3Δ200 leu2Δ0 met15Δ0 hnt2Δ::kanMX2*	BY71 segregant, this work
BY71-16d	*MAT***a** *his3Δ200 leu2Δ0 met15Δ0*	BY71 segregant, this work

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 nondisrupted *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 effect of *hnt2* disruption on ApppN accumulation. *hnt2Δ* 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 phosphorylases 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,

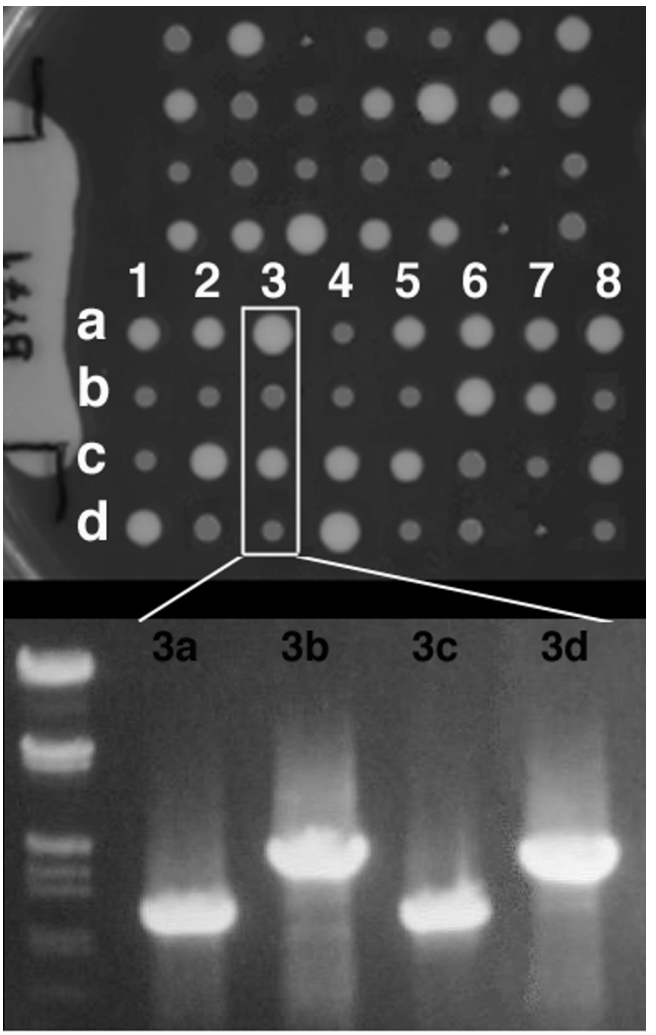

Figure 1
Disruption of *hnt2*. Strain BY16 was crossed with strain BY4717 to generate diploid strain BY71, which was dissected to generate haploid progeny. Markers were scored for mating type, auxotrophic requirements and G418-resistance. The two small segregants per tetrad are *ade2Δ* mutants. Here, four segregants that had been scored for G418-resistance were scored for size of the *HNT2* locus by PCR using diagnostic primers 4722 and 4726. G418-sensitive progeny, BY71-3a and BY71-3c produced a product of 1200 bp while G418-resistant progeny BY71-3b and BY71-3d produced a product of 1976 bp, demonstrating physical linkage of *kanMX2* to *hnt2* disruption.

were expressed from the *HNT2* promoter on plasmids (Table 3) in *hnt2ΔADE2* 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 ^{3}H 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 $CdCl_2$, 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 ApppN 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 $CdCl_2$ 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

Table 2: Intracellular concentration (μM) of dinucleoside polyphosphates in segregants from three complete BY71 tetrads

Culture Time	24 hr		48 hr		72 hr		
Nucleotide	ApppN	AppppN	ApppN	AppppN	ApppN	AppppN	
Segregant							Relevant Genotype
1a	5.96	0.27	112	0.26	251	0.19	*hnt2Δ ADE2*
1b	14.2	0.11	42.7	0.12	101	0.16	*hnt2Δ ade2*
1c	1.44	0.16	2.86	0.09	2.50	0.09	*HNT2 ade2*
1d	0.26	0.04	1.07	0.38	0.60	0.16	*HNT2 ADE2*
2a	42.6	0.23	86.6	0.19	102	0.18	*hnt2Δ ade2*
2b	39.1	0.37	294	1.51	346	0.26	*hnt2Δ ADE2*
2c	0.29	0.06	0.40	0.05	0.36	0.05	*HNT2 ade2*
2d	2.59	0.17	0.86	0.21	0.57	0.07	*HNT2 ADE2*
4a	21.6	0.52	160	0.37	200	0.42	*hnt2Δ ADE2*
4b	0.51	0.09	0.24	0.002	0.09	0.001	*HNT2 ADE2*
4c	0.34	0.07	0.95	0.03	0.80	0.06	*HNT2 ade2*
4d	8.21	0.10	26.7	0.07	52.1	0.19	*hnt2Δ ade2*

Table 3: Plasmids used in this study

Name	Features	Background and source
pRS423	YEp *HIS3*	[39]
pB05	YEp *HIS3 HNT2*	pRS423, this work
pB32	YEp *HIS3 HNT2-His109Ala*	pB05, this work
pB86	YEp *HIS3 HNT2-His109Asp*	pB05, this work
pM1	YEp *HIS3 KRS1*	pRS423, this work

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 *hnt2ΔADE2* 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 $CdCl_2$ or 46°C heat shock and intracellular concentrations of ApppN and AppppN were determined at 30-minute timepoints.

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 $CdCl_2$. 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 ApppN (~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

Table 4: Intracellular concentration (μM) of dinucleoside polyphosphates controlled by the Hnt2 active site

Culture time	24 hr		48 hr		
Nucleotide	ApppN	AppppN	ApppN	AppppN	
Plasmid in strain BY71-6c					Genotype
None	20.70	0.16	129.00	2.16	*hnt2Δ*
pRS423	23.80	0.21	140.00	1.11	*hnt2Δ*
pB05	0.51	0.07	1.11	0.32	*HNT2*
pB32	18.70	0.17	201.00	1.69	*HNT2-His109Ala*
pB86	13.40	0.16	80.40	1.00	*HNT2-His109Asp*

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

Nucleotide	ApppN			AppppN		
Culture Time	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
Relevant Genotype						
HNT2	0.52	1.60	0.22	0.29	0.19	0.02
HNT2 YEp*KRS1*	1.28	1.29	0.20	0.34	0.22	0.01
hnt2Δ	6.25	14.10	3.54	0.35	0.26	0.02
hnt2Δ YEp*KRS1*	5.83	15.50	4.20	0.40	0.30	0.01

His96 is the nucleophile that attacks the α-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* had phenotypic consequences. In the case of the Hint-homologous *HNT1* gene of *S. cerevisiae*, the phenotype of the single mutant was mild and the biological pathway, positive regulation of Kin28, the yeast homolog of Cdk7, was revealed by synthetic lethal interactions between *hnt1* and hypomorphic alleles of *cak1*, *kin28*, *ccl1* and *tfb3*[9]. Because backup systems to limit problems with *hnt2* mutant cells apparently exist, synthetic lethal interactions may be critical to identify the Hnt2 biological pathway. Thus, if the major function of *HNT2* is simply control of dinucleoside polyphosphates lest their accumulation inhibit other enzymes (i.e., McLennan's "foe" hypothesis [42]), then synthetic lethal phenotypes ought to be sought with *hnt2*mutant strains that are wild-type for the adenine biosynthetic pathway, potentially under heat shock conditions. On the other hand, if the major function of Hnt2 depends on formation of an enzyme-substrate complex [16], then synthetic lethal interactions ought to be sought in heat shocked *ADE2 HNT2-His109Ala* strains. In fact, because *ADE2 HNT2-His109Ala* strains have high levels of dinucleoside polyphosphates and an Hnt2 polypeptide, such a strain may be sensitized to secondary mutations, whether dinucleoside polyphosphates are friend or foe [42].

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].

Table 6: Intracellular concentration (μM) of ApppN as a function of stress treatments, *HNT2* genotype, and moderate overexpression of lysyl aminoacyl-tRNA synthetase

Relevant Genotype	-his	1 M sorbitol	10 mM caffeine	2 mM $CdCl_2$	46°C	H_2O	YPD
HNT2	0.58 ± 0.36	1.19 ± 0.37	1.15 ± 0.12	1.12 ± 0.29	2.16 ± 0.10	0.54 ± 0.12	0.44 ± 0.09
HNT2 YEp*KRS1*	0.60 ± 0.51	0.84 ± 0.21	2.74 ± 1.37	1.14 ± 0.17	2.11 ± 0.25	0.49 ± 0.05	0.41 ± 0.13
hnt2Δ	10.7 ± 7.51	6.65 ± 0.82	12.5 ± 3.91	4.92 ± 2.67	44.2 ± 7.51	5.92 ± 1.66	5.97 ± 1.38
hnt2Δ YEp*KRS1*	19.0 ± 8.21	6.36 ± 2.10	3.84 ± 1.05	11.6 ± 4.36	40.6 ± 5.62	8.82 ± 3.57	1.40 ± 0.52

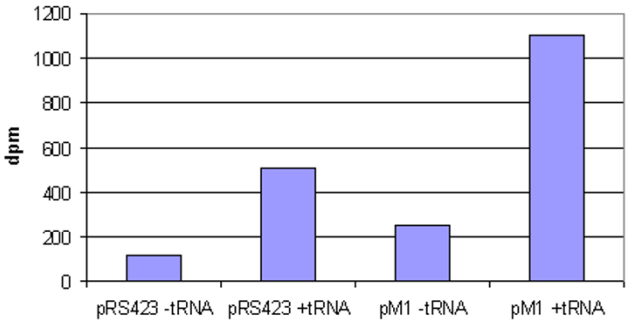

Figure 2
Moderate overexpression of lysyl tRNA synthetase activity via multicopy plasmid pM1 Lysates from strain BY71-6c transformants with control plasmid pRS423 and multicopy *KRS1* plasmid pM1 were assayed for incorporation of ^3H lysine in the absence and presence of added yeast tRNA.

Disruption of hnt2

A 1570 bp DNA fragment containing an *hnt2Δ::kanMX2* disruption cassette was generated as described [46]. Primers 4716 (5'GAAGCTCCATTGATCTATCTTGGGCTCAGAATGATCT TAAGCAAAACAAAGCTTCGTACGCTGCAG) and 4717 (5'CGTAAGTATGAATCTATTATTTATTGAACTATAGTGT-TAAACCAGGGCCACTAGTGGATCTGA) were used to amplify the yeast expressible geneticin-resistance gene from pFA6a-*kanMX2*[46] with 50 bp DNA ends corresponding to sequences upstream and downstream of *HNT2*. The resulting fragment was transformed into haploid *S. cerevisiae* strain BY4727, and transformants were selected on YPD with 400 μg/ml geneticin. To ensure that geneticin-resistance was linked to *hnt2* disruption, genomic DNA from several geneticin-resistant colonies was analyzed by diagnostic PCR primers 4726 (5'TCGCT-GATTTGGTAGTCTC) and 4722 (5'GAGTCTCCTCGAG-GAAAG). A transformant shown to contain the 1976 bp *hnt2Δ::kanMX2* product in place of the wild-type 1200 bp *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.

Plasmid constructions

The *HNT2* gene was amplified from genomic DNA of yeast strain SEY6210 [48] with primers PB1 (5'GCAGCG-GATCCTTGGGAT) that spanned a *Bam*HI site upstream of the promoter and PB2 (5'GAGTCTCCTCGAGGAAAG) that spanned a *Xho*I site downstream of the terminator. The 1316 bp *Bam*HI-*Xho*I fragment containing *HNT2* was ligated to *Bam*HI and *Xho*I-cleaved plasmid pRS423 [39] to generate plasmid pB05. Plasmids pB32 and pB86, carrying H109A and H109D alleles of *HNT2*, were constructed by site-directed mutagenesis [49] of plasmid pB05 using primers PB3 (5'ATAATGTGTGTAGCCAAGT-GGGGT) and PB4 (5'TAATGTGTGTATCCAAGTGGGG-TAC). The *S. cerevisiae* gene encoding lysyl tRNA synthetase (*KRS1*) was amplified as a 2.9 kbp genomic fragment from strain SEY6210 using primers MR20 (5'CGAGCTCGGTTGGA TGACTTTAAAATGACTAAGTTT-GTAGTATCCTCTTTGC ATACTC) and MR21 (5'TCCCCCGGGGGAGCTCCTTTAGGGCTACCGAACAT-AAACAAATTTAGGTAA TGAGTTTTC). This product, cloned into the *Sma*I restriction site of plasmid pRS423, generated plasmid pM1 in which *KRS1* is oriented *anti* to *HIS3*. Plasmids are summarized in Table 3.

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 ApppN and AppppN 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

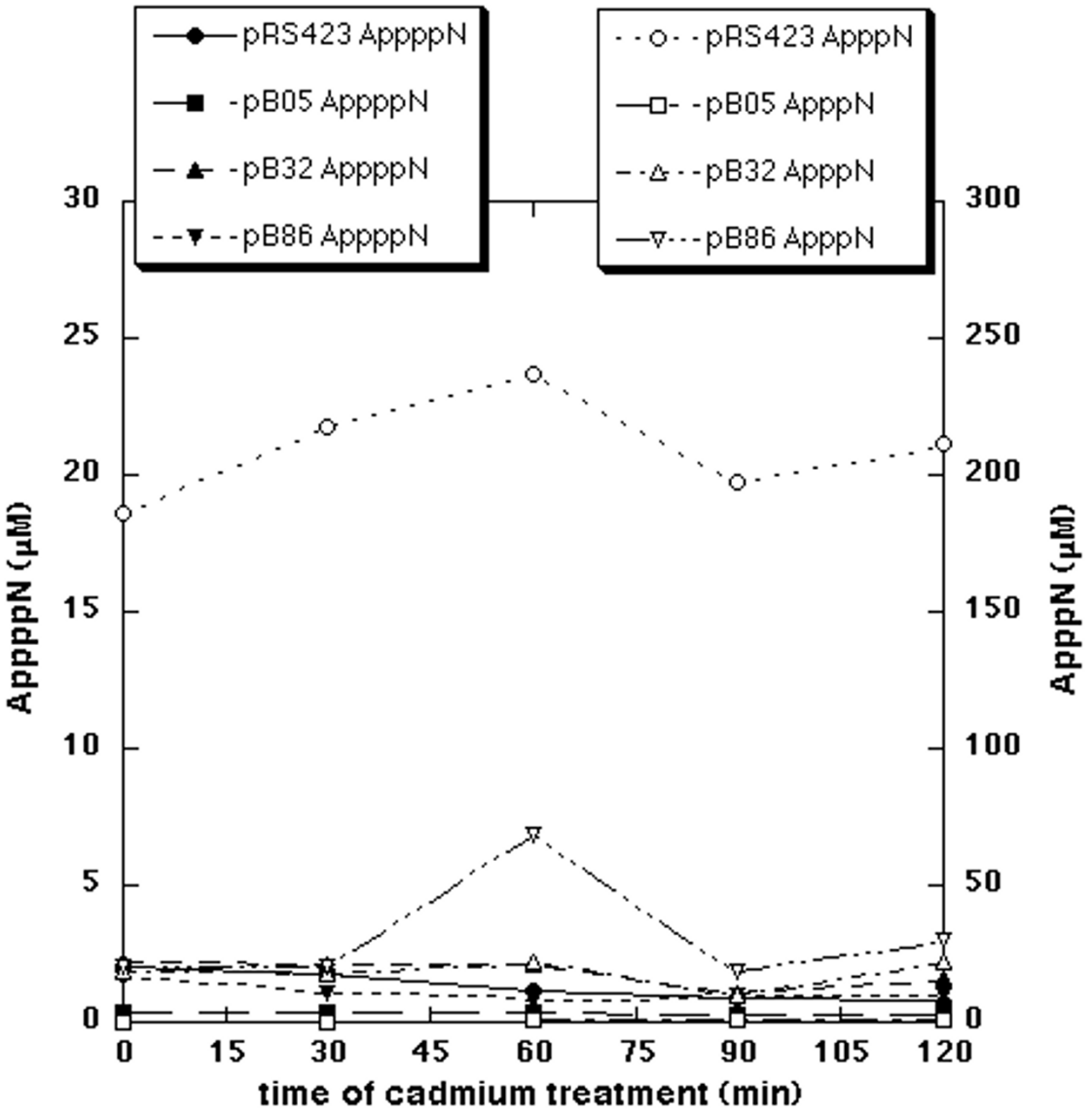

Figure 3
Cadmium is a poor inducer of dinucleoside polyphosphates Calculated intracellular concentrations of ApppN and AppppN of transformants of strain BY71-6c as a function of time in 2 mM $CdCl_2$.

media as above. To determine whether a multicopy lysyl tRNA synthetase plasmid affected accumulation of dinucleoside polyphosphates, we transformed *HNT2 ADE2* strain BY71-16d and *hnt2ΔADE2* 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 *hnt2Δ* and *hnt2Δ* YEp*KRS1*, respectively) and strain BY71-16d was transformed with the same plasmids (effectively *HNT2Δ* and *HNT2Δ* YEp*KRS1*, 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

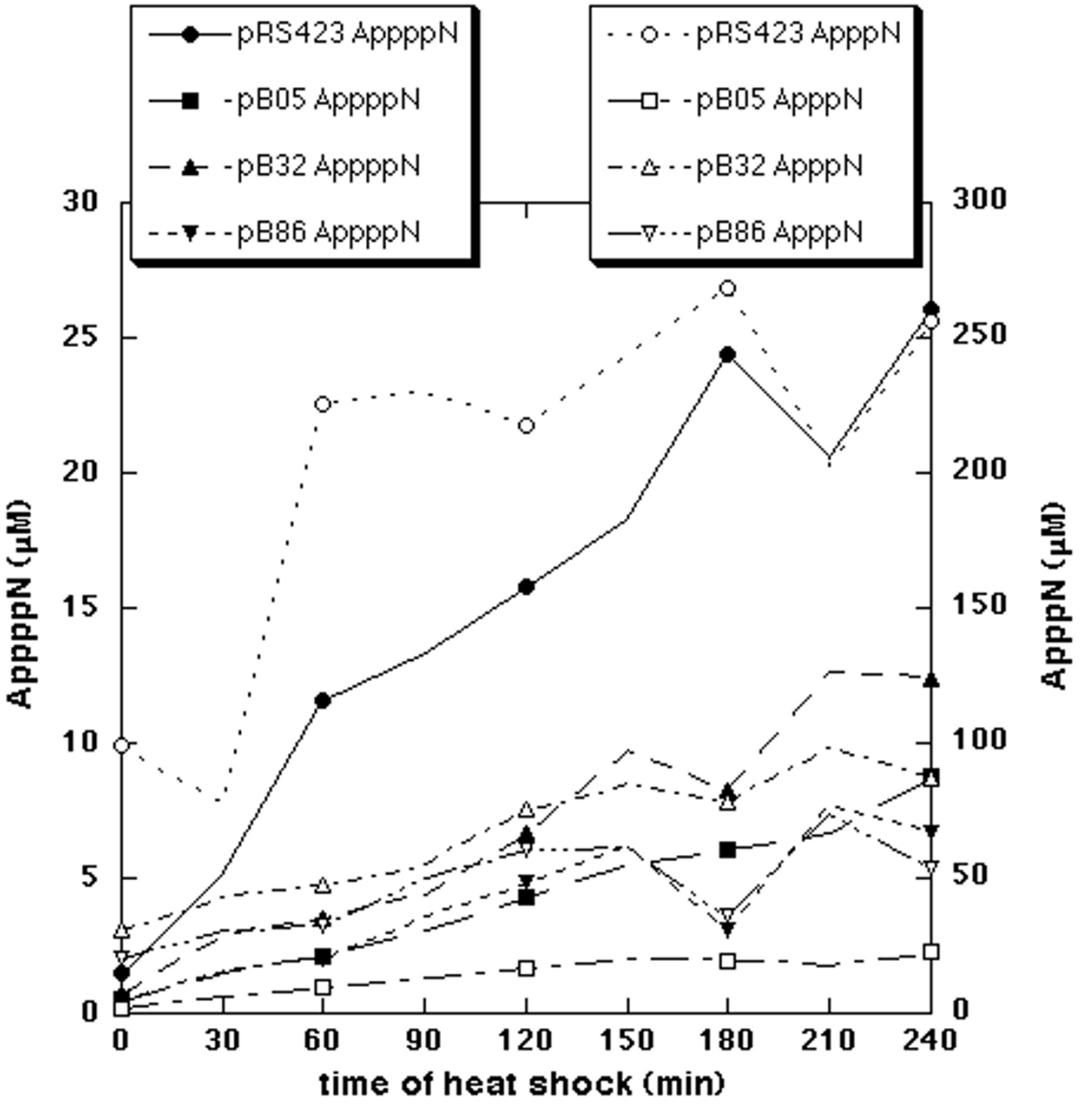

Figure 4
Heat shock induces dinucleoside polyphosphates Calculated intracellular concentrations of ApppN and AppppN of transformants of strain BY71-6c as a function of time at 46°C.

other samples were incubated for 2 hours at room temperature 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 transformed with pRS423, pB05, pB32, or pB86. Transformants were cultured for 48 hours, treated with 2 mM $CdCl_2$ or 46°C heat shock, and then harvested for nucleotide quantitation at 30 minute intervals. Experiments presented 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 culture, generating a higher or lower range of values in independently conducted experiments, we did not average values obtained in separate experiments. For the data presented in Table 6, triplicate cultures were prepared and the

ApppN 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 $MgCl_2$, 4 mM dithiothreitol, 2 mM ATP, 50 mM Na HEPES pH 7.5, 20 μM lysine (10 μCi ^{3}H 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% trichloroacetic acid twice, washed twice in 95% ethanol, rinsed in ether and scintillation-counted.

Authors' contributions

Dr. Bieganowski constructed most of the initial plasmids and strains for this study. Drs. Rubio-Texeira and Dr. Varnum performed the experiments described in this work in consultation with Dr. Brenner, who proposed the experimental questions. Drs. Rubio-Texeira, Varnum and Brenner wrote the manuscript, which was read and approved by each author.

Authors' note

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.

Acknowledgements

This work was supported by a grant from the National Cancer Institute (CA75954). We thank Dr. Ya-Ming Hou and her laboratory for guidance in measuring lysyl tRNA synthetase activity and an anonymous reviewer for helpful suggestions.

References

1. Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Druck T, *et al*: **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
2. Pekarsky Y, Campiglio M, Siprashvili Z, Druck T, Sedkov Y, Tillib S, Draganescu A, Wermuth P, Rothman JH, Huebner K, *et al*: **Nitrilase and Fhit homologs are encoded as fusion proteins in Drosophila melanogaster and Caenorhabditis elegans.** *Proc Natl Acad Sci USA* 1998, **95:**8744-8749
3. Glover TW, Hoge AW, Miller DE, Ascara-Wilke JE, Adam AN, Dagenais SL, Wilke CM, Dierick HA, Beer DG: **The murine Fhit gene is highly similar to its human orthologue and maps to a common fragile site region.** *Cancer Res* 1998, **58:**3409-14
4. Fong LYY, Fidanza V, Zanesi N, Lock LF, Siracusa LD, Mancini R, Siprashvili Z, Ottey M, Martin SE, Dolsky R, *et al*: **Muir-Torre-like syndrome in *FHIT* deficient mice.** *Proc Natl Acad Sci USA* 2000, **97:**4742-4747
5. Dumon KR, Ishii H, Fong LYY, Zanesi N, Fidanza V, Mancini R, Vecchione A, Baffa R, Trapasso F, During MJ, *et al*: **FHIT gene therapy prevents tumor development in Fhit-deficient mice.** *Proc Natl Acad Sci USA* 2001, **98:**3346-3351
6. Brenner C, Bieganowski P, Pace HC, Huebner K: **The histidine triad superfamily of nucleotide-binding proteins.** *J Cell Physiol* 1999, **181:**179-187
7. 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:**
8. Brenner C, Garrison P, Gilmour J, Peisach D, Ringe D, Petsko GA, Lowenstein JM: **Crystal structures of Hint demonstrate that histidine triad proteins are GalT-related nucleotide-binding proteins.** *Nat Struct Biol* 1997, **4:**231-238
9. Bieganowski 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
10. Date H, Onodera O, Tanaka H, Iwabuchi K, Uekawa K, Igarashi S, Koike R, Hiroi T, Yuasa T, Awaya Y, *et al*: **Early-onset ataxia with ocular motor apraxia and hypoalbuminemia is caused by mutations in a new HIT superfamily gene.** *Nat Genet* 2001, **29:**184-8
11. Moreira MC, Barbot C, Tachi N, Kozuka N, Uchida E, Gibson T, Mendonca P, Costa M, Barros J, Yanagisawa T, *et al*: **The gene mutated in ataxia-ocular apraxia 1 encodes the new HIT/Zn-finger protein aprataxin.** *Nat Genet* 2001, **29:**189-93
12. Huang Y, Garrison PN, Barnes LD: **Cloning of the *Schizosaccharomyces pombe* gene encoding diadenosine 5',5-P1,P4-tetraphosphate (Ap4A) asymmetrical hydrolase: sequence similarity with the histidine triad (HIT) protein family.** *Biochem J* 1995, **312:**925-932
13. Chen J, Brevet A, Blanquet S, Plateau P: **Control of 5',5'-dinucleoside triphosphate catabolism by APH1, a Saccharomyces cerevisiae analog of human FHIT.** *J Bacteriol* 1998, **180:**2345-9
14. 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'''-P-1,P-3-triphosphate hydrolase.** *Biochemistry* 1996, **35:**11529-11535
15. Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganowski 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:**907-17
16. Pace HC, Garrison PN, Robinson AK, Barnes LD, Draganescu A, Rosler A, Blackburn GM, Siprashvili Z, Croce CM, Huebner K, *et al*: **Genetic, biochemical, and crystallographic characterization of Fhit-substrate complexes as the active signaling form of Fhit.** *Proc Natl Acad Sci USA* 1998, **95:**5484-9
17. Siprashvili Z, Sozzi G, Barnes LD, McCue P, Robinson AK, Eryomin V, Sard L, Tagliabue E, Greco A, Fusetti L, *et al*: **Replacement of FHIT in cancer cells suppresses tumorigenicity.** *Proc Natl Acad Sci USA* 1997, **94:**13771-13776
18. Werner NS, Siprashvili Z, Fong LYY, Marquitan G, Schroder JK, Bardenheuer W, Seeber S, Huebner K, Schutte J, Opalka B: **Constitutive expression of exogenous Fhit in renal carcinoma cell lines confers differential tumor suppression.** *Cancer Res* 2000, **60:**2780-2785
19. Draganescu A, Hodawadekar SC, Gee KR, Brenner C: **Fhit-nucleotide specificity probed with novel fluorescent and fluorigenic substrates.** *J Biol Chem* 2000, **275:**4555-4560
20. Robinson AK, de la Pena CE, Barnes LD: **Isolation and characterization of diadenosine tetraphosphate (Ap4A) hydrolase from Schizosaccharomyces pombe.** *Biochim Biophys Acta* 1993, **1161:**139-148
21. 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:**2342-4
22. Ingram SW, Barnes LD: **Disruption and overexpression of the Schizosaccharomyces pombe aph1 gene and the effects on intracellular diadenosine 5',5'''-P1, P4-tetraphosphate (Ap4A), ATP and ADP concentrations.** *Biochem J* 2000, **350:**663-9
23. Tanner JA, Abowath A, Miller AD: **Isothermal Titration Calorimetry Reveals a Zinc Ion as an Atomic Switch in the Diadenosine Polyphosphates.** *J Biol Chem* 2002, **277:**3073-3078

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 adenylylated nucleotides are synthesized as a consequence of oxidation 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:**16459-65
31. Coste H, Brevet A, Plateau P, Blanquet S: **Non-adenylylated 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:**6892-9
35. Avila DM, Robinson AK, Kaushal V, Barnes LD: **A paradoxical increase of a metabolite upon increased expression of its catabolic 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, LÈvque 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. Mirande 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. Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P: **Multifunctional 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 phosphorous-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, Schiestl 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, Philippsen P: **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 cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications.** *Yeast* 1998, **14:**115-32
48. Robinson JS, Klionsky DJ, Banta LM, Emr SD: **Protein sorting in *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and processing of multiple vacuolar hydrolases.** *Mol Cell Biol* 1988, **8:**4936-4948
49. Kunkel TA, Roberts JD, Zakour RA: **Rapid and efficient site-specific mutagenesis without phenotypic selection.** *Methods Enzymol* 1987, **154:**367-382
50. Sherman F: **Getting started with yeast.** *Methods Enzymol* 1991, **194:**3-21
51. Hou YM, Westhof E, Giege R: **An unusual RNA tertiary interaction has a role for the specific aminoacylation of a transfer RNA.** *Proc Natl Acad Sci USA* 1993, **90:**6776-80