

Adenosine Monophosphoramidase Activity of Hint and Hnt1 Supports Function of Kin28, Ccl1, and Tfb3*[§]

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The histidine triad superfamily of nucleotide hydrolases and nucleotide transferases consists of a branch of proteins related to Hint and Aprataxin, a branch of Fhit-related hydrolases, and a branch of galactose-1-phosphate uridylyltransferase (GalT)-related transferases. Although substrates of Fhit and GalT are known and consequences of mutations in Aprataxin, Fhit, and GalT are known, good substrates had not been reported for any member of the Hint branch, and mutational consequences were unknown for Hint orthologs, which are the most ancient and widespread proteins in the Hint branch and in the histidine triad superfamily. Here we show that rabbit and yeast Hint hydrolyze the natural product adenosine-5'-monophosphoramidate (AMPNH₂) in an active-site-dependent manner at second order rates exceeding 1,000,000 M⁻¹ s⁻¹. Yeast strains constructed with specific loss of the Hnt1 active site fail to grow on galactose at elevated temperatures. Loss of Hnt1 enzyme activity also leads to hypersensitivity to mutations in Ccl1, Tfb3, and Kin28, which constitute the TFIIF kinase subcomplex of general transcription factor TFIIF and to mutations in Cak1, which phosphorylates Kin28. The target of Hnt1 regulation in this pathway was shown to be downstream of Cak1 and not to affect stability of Kin28 monomers. Functional complementation of all Hnt1 phenotypes was provided by rabbit Hint, which is only 22% identical to yeast Hnt1 but has very similar adenosine monophosphoramidase activity.

Histidine triad (HIT)¹ proteins are a superfamily of nucleotide-binding proteins named for a near C-terminal HXHXHXX motif (X is a hydrophobic amino acid) positioned at the α -phosphate of nucleotide substrates (1). The first branch of the superfamily is named for rabbit Hint, which had been purified as an abundant protein from cardiac cytosol by adenosine affinity

chromatography (2) and shown to have homologs in all forms of life (1). Recently, Aprataxin, a gene located at 9p13 that is inactivated in ataxia with oculomotor apraxia, the second most common of the autosomal recessive ataxias, was identified as a member of the Hint branch of the HIT superfamily (3, 4). Human Fhit (5), which functions as a tumor suppressor protein in human (6–9) and murine (10, 11) epithelial tissues, is the prototypical member of the second branch of the HIT superfamily. Fhit homologs have been found in fungi (12) and animals (13–16) and exhibit diadenosine polyphosphate hydrolase activity. A third branch of the HIT superfamily contains more distantly related nucleotide transferases including galactose-1-phosphate uridylyltransferase, which is the enzyme deficient in galactosemics (1), budding yeast diadenosine tetraphosphate phosphorylases ApaI and Apa2 (17), and adenylylsulfate:phosphate adenylyltransferase (18). Ironically, although Hint is the most ancient and widespread of the HIT proteins, reasonable Hint substrates remained unidentified, and the consequences of mutations in Hint or Hint orthologs were unknown (19).

Recently, human Hint was identified as a two-hybrid partner of Cdk7, and evidence was presented for a genetic interaction between yeast Hnt1 and a yeast Cdk7 homolog, Kin28 (20). The putative physical interaction between Hint/Hnt1 and Cdk7/Kin28 and the fact that *hnt1* deletion lowers the restrictive temperature of a temperature-sensitive mutation in *kin28* were interpreted to suggest that Hint/Hnt1 alters Cdk7/Kin28 substrate specificity by physical association (20). Cdk7 is a cyclin-dependent kinase that associates with cyclin H (21) and MAT1 (22, 23) to form the mammalian cyclin-dependent kinase activating kinase activity (CAK). The ternary CAK complex is, in turn, part of a general transcription factor TFIIF that contains the catalytic activity to phosphorylate C-terminal heptad repeats of RNA polymerase II large subunit (24, 25). This activity, termed C-terminal domain kinase, is required at post-initiation steps in TFIIF function (26). In yeast, CAK and RNA polymerase II C-terminal domain kinase activities are separated not only by virtue of different requirements for regulatory subunits but also by virtue of different catalytic subunits. In *Saccharomyces cerevisiae*, the CAK for Cdc28 is encoded by monomeric Cak1 (27–29), whereas the major RNA polymerase II C-terminal domain kinase of yeast is Kin28, which is TFIIF-associated (30, 31) and lacks CAK activity (32). In addition to phosphorylating the activation loop of Cdc28, Cak1 phosphorylates the activation loop of Kin28 (33, 34). Because the CAK, Cak1, and RNA polymerase II C-terminal domain kinase, Kin28, could both be considered orthologous to Cdk7 and because Kin28 is a substrate of Cak1, we considered the two-hybrid and yeast genetic data (20) to be equivocal in identifying the direct target of Hnt1 regulation in yeast. Furthermore, we were interested in determining whether an enzymatic or a

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains Table II.

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¹ The abbreviations used are: HIT, histidine triad; CAK, cyclin-dependent kinase activating kinase; AMPNH₂, adenosine-5'-monophosphoramidate; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; HA, hemagglutinin; PIPES, 1,4-piperazinediethanesulfonic acid.

nonenzymatic property of Hnt1 is responsible for Cak1 or Kin28 regulation. To avoid species-specific differences in terminology, we refer to the kinase subcomplex of TFIIF, *i.e.* the yeast Kin28-Ccl1-Tfb3 complex and the mammalian Cdk7-cyclin H-MAT1 complex, as TFIIF (30, 31).

In this study we discover that loss of *hnt1* alone produces a temperature-sensitive phenotype on galactose media and that *hnt1* deficiency leads to synthetic loss of viability with hypomorphic alleles of *cak1*, *ccl1*, and *tfb3*. Additionally, *hnt1* produces synthetic phenotypes with all temperature-sensitive alleles of Kin28 examined, with nonphosphorylatable Kin28, and with Kin28 overexpression. Despite the report of a physical interaction (20), none of the phenotypes correlate with the absence of Hnt1 protein but, rather, with loss of Hnt1 enzymatic activity. We also demonstrate that Hnt1 and mammalian Hint are enzymes that hydrolyze unusual adenosine nucleotides such as AMPNH₂ to AMP plus a presumptive ammonia leaving group and that the active-site histidine we show biochemically to be required for AMPNH₂ hydrolysis is required for biological function and not required for protein stability. Though rabbit Hint is only 22% identical to yeast Hnt1, the enzymatic activity of Hint is substantially the same as that of Hnt1, and expression of the rabbit enzyme fully suppressed all *hnt1Δ* phenotypes. These data suggested that accumulation of an Hnt1 substrate in *hnt1* deletion strains, likely an unusual adenosine mononucleotide and/or a nucleotidylated protein substrate, may inhibit function of Cak1 or Kin28 such that either protein kinase cannot tolerate mutational destabilization in itself or the other molecule. Though Cak1 has an unusual ATP-binding site, we show that strains without Cak1 are strongly Hnt1 and Hnt1 active-site-dependent, and thus, Cak1 is not the target of Hnt1 regulation. Thus, the findings that Hnt1 enzyme activity is limiting for growth of cells with increased Kin28 abundance, reduced Kin28 phosphorylation, and destabilization of Kin28 or the additional TFIIF components Ccl1 and Tfb3 reveal an unanticipated nucleotide-dependent form of Kin28 regulation.

EXPERIMENTAL PROCEDURES

Yeast Molecular Biology and Strain Constructions—Standard yeast media, growth conditions, and genetic manipulations were used (35). Galactose-containing media (Gal) contained 2% galactose, whereas galactose plus raffinose-containing media (GalRaf) contained in addition 1% raffinose. To create diploid yeast strains heterozygous for *hnt1* deletion, primers 4748 (primer sequences are provided in Table II, which is available as supplemental material) and 4749 were used to amplify a *TRP1* fragment (plasmid pRS414 (36) as template) and a *URA3* fragment (plasmid pRS416 (36) as template) with *HNT1* homologous ends and transformed into wild-type diploid strain SEY6210.5 (37). Transformants were selected on synthetic dextrose complete (SDC) –trp media and SDC –ura media and screened using diagnostic PCR primers HNT1–5 and HNT1–3. A trp⁺ isolate heterozygous for *hnt1* disruption named BY1 and a ura⁺ isolate heterozygous for *hnt1* disruption named BY8 were allowed to sporulate, and tetrads were dissected, generating haploid *hnt1Δ* progeny including BY1-2a, BY8-3b, BY8-4a, and BY8-5c. Additionally, a *hnt1Δ::kanMX4* PCR product generated with primers 4748 and 4749 and plasmid pRS400 as template (36) was used to transform haploid wild-type *MATα* wild-type strain GF312-17c (28) and *MATα cdc28-4* strain GF2412 to geneticin resistance to produce *hnt1Δ::kanMX4* strain BY158 and *hnt1Δ::kanMX4 cdc28-4* strain BY169.

Strain BY8-3b was crossed with *kin28-ts2* strain JGV117, *kin28-ts3* strain JGV105, *kin28-ts7* strain JGV111, and *kin28-ts8* strain JGV112 (38) to generate four strains heterozygous for *hnt1Δ* and *kin28* alleles named BK2, BK3, BK7, and BK8, respectively, which were dissected to yield *hnt1Δ kin28* double mutant haploid progeny including BK2-2a, BK3-8b, BK7-1c, and BK8-2a.

To introduce *hnt1Δ* into the *cak1-civ1ts4* background, *hnt1Δ* strain BY158 was mated with *cak1* strain GF2351 (28), and a double mutant strain BY161 was obtained by tetrad dissection. This strain was backcrossed to wild-type strain SEY6210 (37), and strain BY164 of genotype *hnt1Δ cak1-civ1ts4* was obtained by tetrad dissection.

To generate strain BY185 of genotype *hnt1Δ::kanMX4 cdc28Δ0 cak1Δ0 kin28Δ0* carrying *CDC28-4324* and alleles of *KIN28* on a plasmid, wild-type diploid strain SEY6210.5 (37) was disrupted with a PCR-generated *cdc28Δ::loxP-kanMX-loxP* disruption cassette generated by amplification of plasmid pUG6 (39) with primers 5122 and 5123. The *cdc28Δ::loxP-kanMX-loxP* heterozygous diploid strain BY172 was confirmed by amplification with diagnostic primers 5126 and 5127 and then transformed with plasmid pSH47, which expresses *cre* recombinase from the *GAL1* promoter (39). A geneticin-sensitive derivative of genotype *cdc28Δ0/CDC28* was cured of plasmid pSH47, named strain BY173, and subjected to transplacement of *cak1Δ::loxP-kanMX-loxP* using primers 5124 and 5125. Heterozygosity for the *cak1* disruption in strain BY175 was confirmed with primers 5130 and 5131, and the *cak1Δ0* allele was recovered in strain BY176 as above. *kin28Δ::loxP-kanMX-loxP* replacement of strain BY176 was accomplished with primers 5132 and 5133 and confirmed with primers 5134 and 5135 to generate strain BY177, whose geneticin-sensitive *kin28Δ0* derivative was named strain BY178. This strain was transformed with a YCp*TRP1* plasmid carrying *CDC28-4324* (40) and pB192, which expresses *URA3* and *KIN28-T162A*, and subjected to tetrad dissection. Segregant BY178-6a scored as trp⁺ and ura⁺ and, additionally, 5-fluoroorotic acid-sensitive and contained *cdc28Δ0*, *cak1Δ0* and *kin28Δ0* disruption alleles, as evidenced by diagnostic PCR. This strain was used to derive the *hnt1Δ::kanMX4* disruptant BY185 using primers 4748 and 4749, as confirmed by diagnostic primers HNT1–5 and HNT1–3. *LEU2* plasmids pB164, pB165 and pB183, which express *KIN28*, *KIN28-T162A*, and *KIN28-T162E*, respectively, were then transformed into strain BY185 with eviction of plasmid pB192.

To generate a *ccl1-ts4 hnt1Δ* double mutant and a *tfb3-rig2ts23 hnt1Δ* double mutant, *hnt1Δ* strain BY8-4a was mated with *ccl1-ts4* strain GF2093 (41) and *tfb3-rig2ts23* strain GF2217 (42) to generate diheterozygous diploid strains BK10 and BK13, respectively. Diploids were allowed to sporulate, and tetrads were dissected, yielding double mutant segregants BK10-5a and BK13-1c, respectively.

To generate strain BY155-8c of genotype *hnt1Δ kin28Δ* carrying alleles of *KIN28* on a plasmid, a *kin28Δ::kanMX4* construct was generated with primers 5046 and 5047 and plasmid pRS400 as template (36). Diheterozygous strain BY155, whose genotype was confirmed by diagnostic PCR with primers 5048 and 5049, was then transformed with *URA3 KIN28* plasmid pB192. Isolate BY155-8c was obtained by tetrad dissection and, after transformation with plasmids pB164 or pB165 containing *LEU2* and *KIN28* or *KIN28-T162A*, was cured of plasmid pB192. Haploid strain YGL26 (34) of genotype *kin28Δ*, expressing HA-tagged Kin28 from plasmid pGK13 (32), was used to produce strain BY186 (genotype *hnt1Δ kin28Δ*, expressing HA-tagged Kin28 from plasmid pGK13) by direct transformation with the *hnt1Δ::kanMX4* construct.

Plasmid Constructions—*Escherichia coli* strain XL-1 Blue was used for bacterial cloning and plasmid amplification. Bacterial media and standard molecular biology techniques were as described (43). All plasmid constructions were confirmed by DNA sequencing. To create plasmid pB42 containing *HNT1* for expression in yeast with its endogenous promoter, genomic DNA from strain SEY6210 (37) was amplified using primers 4787 and 4795. The amplified product was cleaved with *Bam*HI and *Kpn*I, and cloned into *Bam*HI and *Kpn*I-digested pRS423 (44) to generate plasmid pB42. Plasmid pB150 was generated by site-directed mutagenesis (45) of pB4 using primer 5052 to generate the *hnt1-H116A* allele. To create plasmid pB159 containing rabbit *HINT* under the control of the *GAL1* promoter, *HINT* cDNA was amplified from pSGA02-*HINT* (2) using primers 5085 and 5086. The product was then digested with *Bam*HI and *Xho*I and cloned into *Bam*HI and *Xho*I-cleaved plasmid pRS425*GAL1* (46). To create plasmid pB216 containing *HNT1* with a C-terminal triple FLAG tag (47) for expression in yeast, *HNT1* was amplified with primers HNT1–5 and 5190, and the *Bam*HI- and *Xho*I-cleaved product was cloned into *Bam*HI- and *Xho*I-cleaved pRS413 (36). To create plasmid pB217 containing the triple FLAG-tagged *hnt1-H116A* allele, site-directed mutagenesis was performed with primer 5052.

To create plasmid pB24 containing the *HNT1* cDNA in plasmid pSGA02 (48) for bacterial expression, primers 4739 and 4740 were used to amplify a product from oligo(dT)-primed yeast cDNA, which was digested with *Nde*I and *Xho*I and cloned into *Nde*I- and *Xho*I-cleaved pSGA02. To create plasmid pB195 containing *HNT1-H116A* for bacterial expression, plasmid pB24 was subjected to site-directed mutagenesis using primer 4766.

To create plasmid pB176, containing *PTC2* under the control of the *GAL1* promoter, genomic DNA from wild-type strain SEY6210 (37) was amplified using primers 5108 and 5109. The amplified product was

digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI- and *Eco*RI-cleaved plasmid pRS426*GAL1* (46).

To create plasmid pB164, containing *KIN28* expressed from its own promoter, genomic DNA from SEY6210 was amplified using primers 5066 and 5067. The product was digested with *Bam*HI and *Eco*RI and cloned into *Bam*HI plus *Eco*RI-cleaved plasmid pRS415 (36). Site-directed mutagenesis of plasmid pB164 using primer 5075 generated plasmid pB165 containing *KIN28-T162A*. Mutagenesis of pB164 using primer 5116 generated pB183 containing *KIN28-T162E*. To construct plasmid pB192, the *Bam*HI-*Xho*I fragment of plasmid pB165 was transferred to *Bam*HI- and *Xho*I-cleaved pRS416 (36). To create plasmid pB153, the *KIN28* cDNA was amplified from oligo(dT)-primed yeast cDNA using primers 5054 and 5055 and inserted into pGEM (Promega). The *KIN28* cDNA was excised from plasmid pB153 with *Spe*I and *Xho*I and inserted into *Spe*I- plus *Xho*I-cleaved pRS425*GAL1* to construct plasmid pB194.

Purification of Recombinant Hint and Hnt1 Proteins—Rabbit and yeast Hint proteins were expressed in *E. coli* from plasmids pSGA02-*HINT* (2), pB24 (*HNT1*), and pB194 (*hnt1-H116A*) according to published procedure (2). Frozen and thawed cell pellets (250 mg wet weight) were lysed in 12.5 ml of buffer A (20 mM Tris, pH 7.5, 150 mM NaCl) with one EDTA-free protease inhibitor mixture tablet (Roche Molecular Biochemicals). Lysates were cleared by centrifugation and further cleared after precipitation with 1 mg of protamine sulfate. Ammonium sulfate was added to 40% saturation, and the 40% ammonium sulfate pellet was dialyzed against buffer A and clarified by centrifugation. Rabbit Hint and yeast Hnt1-His116Ala were purified by AMP-agarose (Sigma) affinity chromatography using a 4-ml column, washing with 24 column volumes of buffer A, and eluting with 3 column volumes of buffer A with 200 μ M adenosine for Hint and 10 mM adenosine for Hnt1-H116A. Yeast Hnt1 was purified on a POROS 20 HQ column (PE Biosystems) using 20 mM Tris, pH 7.5, 2% glycerol as a loading and running buffer, and NaCl as an eluant. Homogenous enzyme preparations were dialyzed against buffer A, concentrated to 10 mg/ml, and stored at -80°C .

Enzymatic Assays—Compounds tested as potential substrates were from Sigma, except AMP-*N*-alanine methyl ester and AMP-*N*- ϵ -(*N*-acetyl lysine methyl ester), which were synthesized according to a modification of the synthesis of AMP-*N*- ϵ -lysine (49), and human DNA ligase I adenylylated intermediate, a gift of Alan E. Tomkinson, which was prepared as described (50). Compounds were screened for hydrolysis in 10- μ l reactions containing 1 mM substrate, 0.5–2.0 μ g of enzyme, 0.5 mM MgCl₂, 20 mM Na-PIPES at pH 7.2 for 5–12 h at 37 $^{\circ}\text{C}$. Reactions repeated without MgCl₂ were within 10% of the values reported. Reactions were diluted to 100 μ l and analyzed by liquid chromatography on a MonoQ HR 5/5 column (Amersham Biosciences, Inc.) with mobile phases that were suitable mixtures of water and 1.25 M NH₄HCO₃, pH 8. Ultraviolet absorbing peaks corresponding to nucleoside monophosphate products were integrated and converted to nmol using standard curves for AMP and ratios of appropriate extinction coefficients to that of AMP. Activity on *p*-nitrophenyl TMP was assayed colorimetrically at 400 nm as described (51). Human DNA ligase I adenylylated intermediate (50 pmol) was treated with 1 μ g of enzyme in 50 μ l of ligase buffer (30 mM Tris-HCl, 50 mM NaCl, 0.5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, pH 7.5) for 1 h at 37 $^{\circ}\text{C}$ and analyzed by liquid chromatography for release of AMP and for the size and retention time of the ligase peak at 280 nm. Compounds that yielded activity greater than 0.005 nmol min⁻¹ μ g⁻¹ were assayed further in 50–200- μ l reactions with 0.2 mM substrate, 0.005–1 μ g of enzyme, and 20 mM PIPES, pH 7.2, for 10 min at 37 $^{\circ}\text{C}$. Reactions were stopped by adding NaOH to adjust the pH to 11, except for ester-containing reactions, which were stopped by freezing on dry ice. For saturation curves, reactions were performed in 200 μ l with 0.5–3 ng of enzyme. In some cases, 10 mM Na-BES or 10 mM imidazole HCl at pH 7.2 was used as buffer because of an interfering peak in PIPES. The low *K_m* of the rabbit enzyme made it necessary to calculate initial rates from reactions that exceeded 10% hydrolysis with corrections as described (52). Thus, the reported *K_m* of 68 nM should be considered an upper limit.

Evaluation of Hnt1 and Kin28 Abundance in Vivo—To examine steady state abundance of FLAG-tagged wild-type and active-site mutant Hnt1 proteins, strain BY8-5c was transformed with empty vector pRS413 and with plasmids pB216 and pB217, which encode the two FLAG-tagged Hnt1 constructs. Transformants were grown at 37 $^{\circ}\text{C}$ in synthetic Gal complete –his media to an optical density (600 nm) of 0.6. Washed cell pellets were frozen at -20°C , and 0.4-g samples of cells were lysed with 0.8 g of glass beads (0.5 mm, Sigma) in 1 ml of cold 150 mM Tris acetate, pH 7.5, 300 mM (NH₄)₂SO₄, 1 mM spermidine, 1 mM dithiothreitol, 10% glycerol, 1 \times Complete protease inhibitor mixture

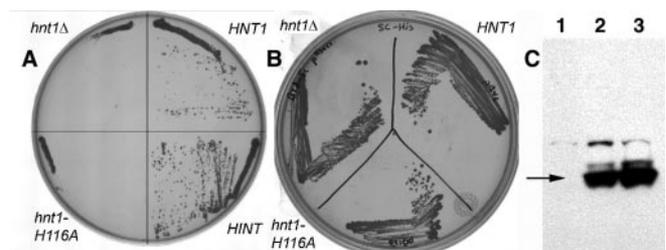


FIG. 1. Hnt1 enzyme activity is necessary for growth on galactose at 39 $^{\circ}\text{C}$ and is complemented by rabbit Hint. **A**, an *hnt1* Δ strain (BY8-5c) was transformed with plasmids containing either *HNT1* (pB42), *hnt1-H116A* (pB150), rabbit *HINT* cDNA under *GAL1* control (pB159), or an empty vector (pRS423), and colonies were grown at 39 $^{\circ}\text{C}$ on synthetic GalRaf complete media for 6 days. **B**, strain BY8-5c transformants containing either *HNT1* (pB42), *hnt1-H116A* (pB150), or empty vector (pRS423) were grown at 39 $^{\circ}\text{C}$ on SDC media for 4 days. **C**, strain BY8-5c transformants containing either empty vector (pRS413, lane 1), FLAG-tagged *HNT1* (pB216, lane 2), or FLAG-tagged *hnt1-His119Ala* (pB217, lane 3) were grown in SGal –his at 37 $^{\circ}\text{C}$ and subjected to a Western blot with anti-FLAG antibody. The lane 1 control demonstrates the specificity of the signal, and an arrow marks the predicted size of FLAG-tagged Hnt1.

(Roche Molecular Biochemicals) with seven 1-min pulses of a Mini-8 Beadbeater (Biospec). Twenty μ g of total protein (determined by the Bio-Rad assay) from the clarified lysate of each sample was electrophoresed (15% SDS-PAGE) and transferred to an Immobilon-P membrane (Millipore). The transfer was probed with a 1:1000 dilution of anti-FLAG M2 monoclonal antibody (Sigma), and the washed blot was probed with a 1:2000 dilution of sheep anti-mouse IgG-horseradish peroxidase conjugate (Amersham Biosciences, Inc.), washed, and developed with ECL reagents (Amersham Biosciences, Inc.) as instructed. To detect HA-tagged Kin28 protein, strains YGL26 and BY186 were grown to an optical density (600 nm) of 0.6 in yeast/peptone/galactose and yeast extract/peptone/dextrose medium at 30, 34, and 37 $^{\circ}\text{C}$. Total soluble protein was extracted as above, electrophoresed (12% SDS-PAGE), and transferred as above. The Western blot was produced with a 1:1000 dilution of ascites fluid from monoclonal antibody HA.11 (Covance) and secondary antibody and detection system as above.

RESULTS

***hnt1* Mutants are gal⁻ at 39 $^{\circ}\text{C}$** —Budding yeast contain one ortholog of *HINT*, *HNT1*, one ortholog of *FHIT*, *HNT2* (1, 19), and one ortholog of the Hint-related enzyme Aprataxin, *HNT3*. To explore the consequences of loss of the *HINT* ortholog, we generated disruption alleles *hnt1* Δ ::*TRP1* and *hnt1* Δ ::*URA3* and introduced them into diploid strains. Diploids heterozygous for *hnt1* Δ were allowed to sporulate, were dissected, and resulted in four viable haploid segregants per tetrad, indicating that *HNT1* is neither required for spore germination nor for viability. Careful characterization of *hnt1* Δ mutants in comparison with isogenic *HNT1* isolates revealed that *hnt1* Δ mutants do not grow on synthetic galactose-containing media at 39 $^{\circ}\text{C}$ (Fig. 1A). Transformation of *hnt1* Δ mutants with a plasmid carrying an intact *HNT1* gene restored full growth on galactose at 39 $^{\circ}\text{C}$, but empty vector did not. As shown in Fig. 1B, *hnt1* Δ mutants display no temperature sensitivity on glucose medium.

Mammalian and Yeast Hint Proteins Possess Adenosine 5'-Monophosphoramidase Activity—Rabbit Hint was initially purified as an abundant cytosolic protein that binds adenosine agarose (2). Crystal structures of Hint bound to GMP, 8-Br-AMP, and adenosine showed that a majority of the residues conserved in the HIT superfamily are located in positions to interact with bound nucleotide and that Hint is structurally related to Fhit and to galactose-1-phosphate uridylyltransferase (1), which are enzymes that utilize a conserved histidine to perform hydrolysis or transfer reactions at the α phosphate of a bound nucleotide (13, 53). It was also reported that human Hint has an extremely weak ADPase activity ($k_{\text{cat}}/K_m = 8.5 \text{ M}^{-1} \text{ s}^{-1}$) (54). Discovery of a physiological assay for yeast Hnt1

TABLE I
Active-site-dependent nucleotide hydrolysis ($\text{nmol min}^{-1} \mu\text{g}^{-1}$) by rabbit Hint and yeast Hnt1

Substrates were assayed at 1 mM unless noted otherwise. Numbers in parentheses are the number of replicate measurements. Error values designate the observed ranges for duplicates and S.D. for $n > 2$.

Substrate	Rabbit Hint	Yeast Hnt1	Hnt1-H116A
AMPNH ₂ (200 μM)	1.71 \pm 0.25 (3)	7.57 \pm 0.97 (4)	<.00003 (4)
AMP- <i>N</i> - ϵ -(<i>N</i> - α -acetyl lysine methyl ester) (200 μM)	1.03 \pm 0.13 (4)	5.92 \pm 1.05 (4)	
AMP- <i>N</i> -alanine methyl ester (200 μM)	0.79 \pm 0.11 (4)	2.70 \pm 0.33 (3)	
AMP morpholidate (200 μM)	0.82 \pm 0.15 (3)	1.37 \pm 0.49 (3)	
AMP α S (200 μM)	0.15 \pm 0.03 (4)	0.020 \pm 0.004 (3)	
AMPSO ₄ (200 μM)	0.075 \pm 0.011 (5)	0.029 \pm 0.005 (4)	
ADP	0.0039 \pm 0.0012 (2)	0.00054 \pm 0.00007 (2)	
GDP	0.0028 \pm 0.0005 (4)	0.00017 \pm 0.00006 (3)	
ADP-ribose	0.0012 \pm 0.0002 (2)	0.00042 \pm 0.00026 (2)	
8-Br-ADP	0.00090 \pm 0.0003 (2)	<0.0001 (2)	
NAD	0.00090 \pm 0.0003 (2)	<0.0001 (2)	
AppA	0.00046 \pm 0.0001 (2)	<0.0001 (2)	
IDP	0.00035 (1)	<0.0001 (1)	
NADH	0.00022 \pm 0.0001 (2)	<0.0001 (2)	
ATP	0.00013 \pm 0.00002 (2)	<0.0001 (2)	
DNA ligase 1 intermediate (1 μM)	<0.0002 (1)	<0.0002 (1)	
<i>p</i> -Nitrophenyl TMP (100 μM)	<0.001 (2)	<0.001 (2)	
AppppppA, ApppppA, AppppA, ApppA, d(pApA), acetylCoA, GTP, GDP-glucose, ITP, UTP, UDP, UDP-glucose, CTP, CDP, 8-Br-ATP	<0.0001 (2)	<0.0001 (2)	

prompted us to explore a range of nucleotide substrates for rabbit and yeast Hint proteins. Rabbit Hint and yeast Hnt1 proteins were expressed in *E. coli* and purified to homogeneity by AMP-agarose affinity or ion exchange chromatography. As shown in Table I, under the conditions of our assay, which could detect as little as 30 fmol of nucleoside monophosphate released/min/ μg of enzyme, ATP was detectable as a weak substrate of rabbit Hint ($130 \text{ fmol min}^{-1} \mu\text{g}^{-1}$) but not yeast Hnt1. Purine nucleotides such as ITP, 8-Br-ATP, GTP, and GDP-glucose, pyrimidine mononucleotides such as CTP, CDP, UTP, UDP, UDP-glucose, and *p*-nitrophenyl TMP, deoxy-pApA, diadenosine polyphosphates ApppA through AppppppA, and acetyl-CoA were not detectably cleaved. Purine nucleoside diphosphates IDP, 8-Br-ADP, GDP and ADP and the smallest purine dinucleoside diphosphate-related compounds such as AppA, NAD, NADH, and ADP-ribose were moderately better substrates than ATP, with adenosine diphosphate showing better cleavage than the other purine nucleoside diphosphates. Incubation with AMP-SO₄ and AMP α S indicated that sulfate and sulfur are much better leaving groups than phosphate for Hint because these compounds were cleaved 20–50-fold better by rabbit and yeast enzymes than ADP.

In a focused search for adenosine mononucleotides with a leaving group other than phosphate, we found that AMP morpholidate, a nucleotide with 5' phosphoramidate (P-N) linkage to a six-membered ring, was cleaved 200-fold better than ADP by rabbit Hint and 2000-fold better than ADP by yeast Hnt1, bringing the hydrolysis rates up to the order of $1 \text{ nmol min}^{-1} \mu\text{g}^{-1}$. Substitution of the morpholine ring with alanine methyl ester or *N*- α -acetyl lysine methyl ester, which maintained a phosphoramidate linkage to AMP, produced substrates about 2-fold better than AMP morpholidate. The smallest possible amine substitution of AMP, AMPNH₂, was the best substrate, consumed at 1.7 and 7.6 $\text{nmol min}^{-1} \mu\text{g}^{-1}$ by rabbit and yeast enzymes, respectively.

We performed initial rate determinations as a function of the concentration of AMPNH₂. As shown in Fig. 2, AMPNH₂ is hydrolyzed to AMP plus a presumptive ammonia leaving group, with k_{cat} and K_m values of $0.20 \pm 0.05 \text{ s}^{-1}$ and $68 \pm 15 \text{ nM}$ ($n = 3$) for the rabbit enzyme and values of $0.87 \pm 0.25 \text{ s}^{-1}$ and $870 \pm 150 \text{ nM}$ ($n = 3$) for the yeast enzyme. To test whether these activities depend on the conserved middle histidine of the

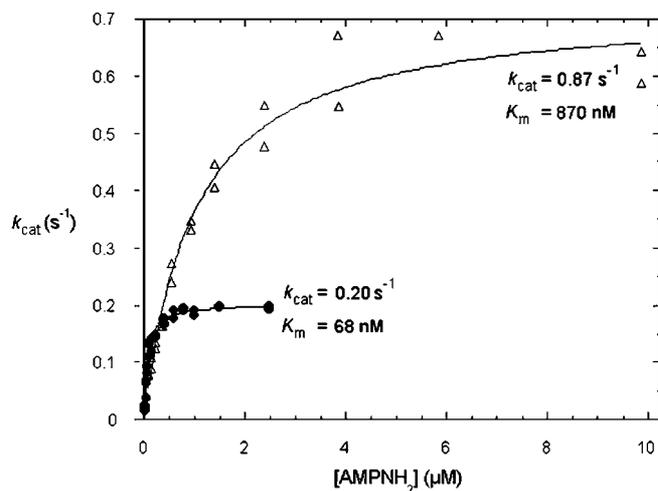


FIG. 2. Hint proteins are adenosine monophosphoramidases. Substrate concentration-dependent rates (per monomer active site) for AMPNH₂ hydrolysis to AMP plus the presumptive ammonia leaving group by rabbit Hint (●) and yeast Hnt1(▲).

HIT motif, which is required for catalytic activity of Fhit (13, 55–57), a H116A mutant form of yeast Hnt1 was generated, purified from *E. coli*, and incubated with the same substrates. No AMP was detected (Table I). Because the high pressure liquid chromatography assay detects enzyme activity at levels 100,000-fold lower than those observed from enzymatic hydrolysis of AMPNH₂, active-site dependence was shown conclusively. Moreover, discovery of AMPNH₂ as a substrate allowed the demonstration that Hint and Hnt1 are more than 100,000-fold more proficient on this substrate (k_{cat}/K_m for rabbit and yeast, $2.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and $1.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively) than on ADP (54).

A review of the literature shows that AMPNH₂ is found in eukaryotic cells (58), is synthesized from AMPSO₄ and ammonia by an enzyme that can be found in a wide variety of bacteria and eukarya (59), and is hydrolyzed to AMP plus ammonia by an enzyme from rat liver that, like Hint, is a homodimer of 14-kDa subunits (60). Because that nucleoside monophosphoramidase activity was reported not to require magnesium (60),

we repeated all Hint and Hnt1 assays without magnesium and found that all metal-free activities were within 10% of the values reported in Table I. The rat liver enzyme was reported to be active on AMP-*N*-aminohexylamine, and the Hint/Hnt1 enzymes are active on the similar substrate AMP-*N*- ϵ -(*N*-acetyl lysine methyl ester) (Table I). Failure to hydrolyze the Lys ϵ amino-linked AMP intermediate of DNA ligase I may be due to steric hindrance of AMP in the ligase active-site cavity (50, 61). Thus, Hint was likely already purified as nucleoside-5'-monophosphoramidase (60). Hint and Hnt1 are postulated to function *in vivo* by hydrolyzing AMPNH₂ or a related substrate, here provisionally termed AMP-X, to AMP plus the protonated leaving group.

Hnt1 Enzymatic Activity Is Necessary for Biological Function and Can Be Functionally Complemented by Rabbit Hint—To test whether Hnt1 enzymatic activity is necessary for complementation of the *hnt1* Δ gal⁻ phenotype at elevated temperatures, the *H116A* allele of *hnt1*, which has no detectable enzymatic activity *in vitro*, was constructed for plasmid expression in yeast under the control of the *HNT1* promoter. As shown in Fig. 1A, *hnt1-H116A* showed no complementation, demonstrating that the active-site histidine residue is necessary for physiological function of the protein. Although the yeast Hnt1-H116A polypeptide had been purified from *E. coli* (above) and did not exhibit reduced steady state abundance *in vivo* or instability as a purified protein, we wished to eliminate the possibility that loss of function of *hnt1-H116A* was caused by protein instability rather than loss of enzymatic activity. We therefore constructed C-terminal triple FLAG-tagged (47) *HNT1* and *hnt1-H116A* alleles and expressed them from the *HNT1* promoter on centromeric plasmids. As expected, FLAG-tagged *HNT1* complemented the gal⁻ phenotype of *hnt1* at 39 °C, whereas FLAG-tagged *hnt1-H116A* did not (not shown). Having validated the constructs, we probed soluble lysates for the presence of the FLAG-tagged antigens at 37 °C, which is the highest temperature at which the FLAG-tagged *hnt1-H116A* strain can be grown. As shown in Fig. 1C, the *hnt1-H116A* allele did not diminish protein expression, and thus, Hnt1 enzymatic activity is necessary for physiological function. The rabbit *HINT* cDNA, encoding a polypeptide with a conserved nucleotide-binding site and, as shown above, conserved enzymatic activity, but retaining only 22% sequence identity to Hnt1 (1), was put under the control of the *GAL1* promoter. Rabbit *HINT* fully suppressed the gal⁻ phenotype of the *hnt1* Δ strain (Fig. 1A).

Loss of Hnt1 Enzymatic Activity Produces Synthetic Loss of Viability with Temperature-sensitive Alleles of *kin28* and *cak1*—Recently it was reported that human Hint has a strong two-hybrid interaction with Cdk7 and that *hnt1* deletion lowers the restrictive temperature of *kin28-ts3* on galactose-containing media (20). These workers also showed a weak two-hybrid interaction between Kin28 and Hnt1 and that a small fraction of overexpressed human Hint could be immunoprecipitated with Cdk7. The results were taken together to suggest that Hint physically associates with Cdk7 and functions to increase substrate specificity for CTD phosphorylation (20). We reasoned that evidence for a physical interaction between Hnt1 and Kin28 could be bolstered if there were allele specificity in the dependence of *kin28-ts* strains for *HNT1*. However, deletion of *hnt1* in strains carrying seven different temperature-sensitive alleles of *kin28* invariably reduced the restrictive temperature on galactose-containing media. Synthetic interactions with four alleles are shown in Fig. 3A. At temperatures permissive for single *kin28-ts* mutations that are selective for the corresponding *kin28-ts hnt1* Δ double mutants, single mutants are slightly enlarged with respect to wild-type cells,

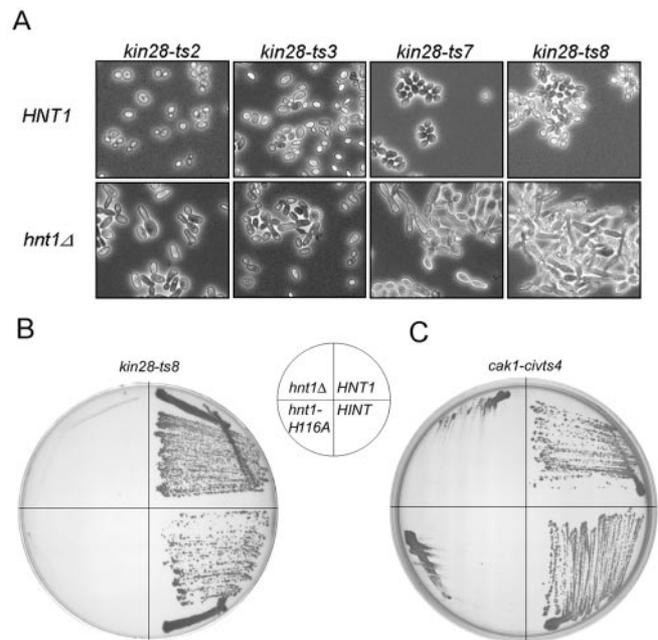


FIG. 3. Synthetic loss of viability between *hnt1* Δ and temperature-sensitive alleles of *kin28* and *cak1*. A, micrographs of strains carrying an *hnt1* deletion with the indicated temperature-sensitive alleles of *kin28* grown in SGalRafC -leu at 30–32 °C, bearing multicopy plasmids containing either wild-type *HNT1* (pB42) or no insert (pRS423). B, an *hnt1* Δ *kin28-ts8* strain (BK8-2a) was transformed with plasmids containing either *HNT1* (pB42), rabbit *HINT* cDNA under *GAL1* control (pB159), *hnt1-H116A* (pB150), or an empty vector (pRS423) and grown at 34 °C on SGalRafC media for 6 days. C, an *hnt1* Δ *cak1-civts4* strain (BY164) was transformed with plasmids containing either *HNT1* (pB42), rabbit *HINT* cDNA under *GAL1* control (pB159), *hnt1-H116A* (pB150), or an empty vector (pRS423), and grown at 34 °C on SGalRafC media for 6 days.

whereas double mutants are highly elongated and cease cell division. To test whether the heightened requirement for Hnt1 of *kin28* mutants reflects an enzymatic or nonenzymatic property of Hnt1, we transformed *hnt1* Δ *kin28-ts8* strain BK8-2a with plasmids expressing wild-type *HNT1*, *hnt1-H116A*, rabbit *HINT*, or with an empty vector. As shown in Fig. 3B, yeast Hnt1 and rabbit Hint proteins were fully functional, whereas yeast Hnt1 with an active-site mutation was without function. Thus, Hnt1 enzymatic activity rather than the Hnt1 polypeptide is conditionally essential in supporting hypomorphic forms of Kin28. Additionally, with weak alleles of *kin28* such as *kin28-ts8*, *hnt1* deletion reduces the restrictive temperature on glucose as well as galactose media (not shown).

To test whether mutation of Cak1, which is not only the CAK for Cdc28 (27–29) but also the CAK for Kin28 (33, 34), also increases the cellular requirement for Hnt1, a strain bearing the *cak1-civts4* allele was disrupted for *hnt1* to generate strain BY164. As with *kin28-ts3*, *cak1* destabilization is synthetically less viable with Hnt1 deletion or ablation of Hnt1 enzymatic activity, and the double mutant phenotype is rescued by expression of rabbit Hint (Fig. 3C). Thus, Hnt1 enzyme activity appears to function as a positive regulator of either Cak1 or Kin28 in yeast, and the Hnt1 requirement is most apparent at elevated temperature on galactose media.

We reasoned that a Hint substrate chemically related to AMPNH₂, provisionally termed AMP-X, may inhibit one of the protein kinases in this pathway. By Occam's Razor, we sought to account for all experimental facts with a single target of Hnt1 regulation, either Cak1 or Kin28, and discover the cellular reasons why mutation of the other kinase also enforces a requirement for Hnt1.

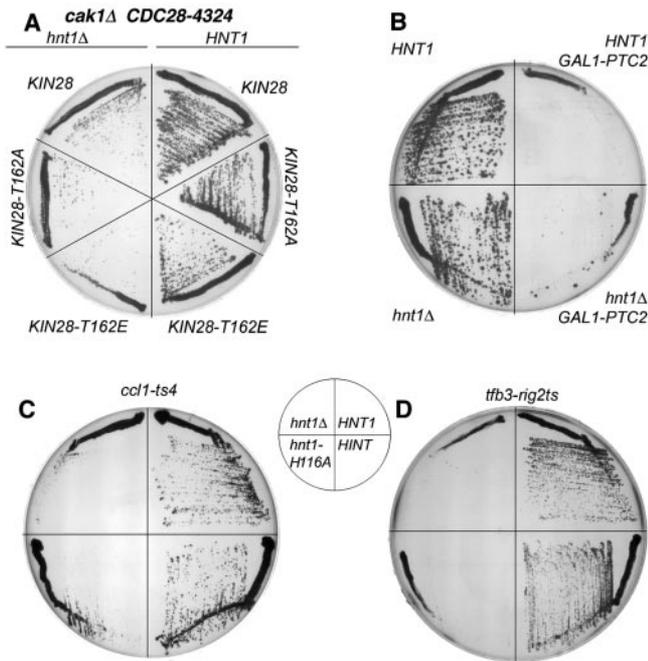


FIG. 4. Cak1 is not the target of Hnt1 regulation, and strains with destabilized Kin28-binding proteins are limited by Hnt1 enzyme activity. **A**, yeast strain BY185 (genotype *hnt1Δ::kanMX4 cdc28Δ0 cak1Δ0 kin28Δ0*, carrying the *cak1*-independent *CDC28-4324* allele on a plasmid and the indicated alleles of *KIN28* on plasmids) was transformed additionally with pRS423 (indicated by *hnt1Δ*) or pB42 (indicated by *HNT1*). Transformants were streaked on SGalC –leu –his media and grown at 30 °C for 5 days. **B**, yeast strain BY1-2a (genotype *hnt1Δ* strain) was transformed with pRS423 (indicated by *hnt1Δ*) or pB42 (indicated by *HNT1*) and with multicopy *GAL1-PTC2* plasmid pB176 or empty vector pRS416. Transformants were grown at 30 °C on SGalRafC –his –ura media for 4 days. **C**, an *hnt1Δ ccl1-ts4* strain (BK10-5a) was transformed with plasmids containing either *HNT1* (pB42), rabbit Hint cDNA (pB159), *hnt1-H116A* (pB150), or an empty vector (pRS423) and grown at 35 °C on SGalRafC for 6 days. **D**, an *hnt1Δ tfb3-rig2ts23* strain (BK13-1c) was transformed with plasmids containing either *HNT1* (pB42), rabbit Hint cDNA (pB159), *hnt1-H116A* (pB150), or an empty vector (pRS423) and grown at 34 °C on SGalRafC media for 6 days.

Cak1 Is Not the Target of Hnt1 Regulation—Because the initial models under consideration propose that AMP-X, an adenosine nucleotide, is a specific inhibitor of a protein kinase, Cak1 as an enzyme with an unusual ATP-binding site (27–29), was a good initial candidate as the direct target of Hnt1 regulation. Specifically, because Cak1 lacks the canonical GXGXXG sequence that nearly all protein kinases use to bind ATP and is uniquely resistant to 5'-fluorosulfonylbenzoyladenine (62), we considered it possible that Cak1 is uniquely sensitive to inhibition by a Hint/Hnt1 nucleotide substrate. Though *CAK1* is an essential gene, it is possible to bypass the essential function with the multiply mutated *CAK1*-independent allele of *CDC28*, *CDC28-4324* (40). Strains containing a *cak1* deletion suppressed by expression of *CDC28-4324* as the sole source of Cdc28 were constructed and then subjected to *hnt1* deletion. If Hnt1 were to function solely as a positive regulator of Cak1, then a strain without Cak1 would not be expected to show Hnt1 dependence. Nonetheless, as shown in Fig. 4A, deletion of *hnt1* produces slow growth in strains without Cak1.

Two additional experiments were inconsistent with Cak1 as the target of Hnt1 regulation. If Cak1 were to be inhibited in *hnt1* cells, the expected consequences would be reduction in Cdc28-activating phosphorylation (27–29) and reduction in Kin28 phosphorylation (33, 34). To determine whether cells with a reduced level of phosphorylated Cdc28 are hyperdependent on Hnt1, we attempted to determine whether *hnt1Δ* pro-

duces synthetic loss of viability with overexpression of Ptc2, a Cdc28 activation-loop phosphatase (63), and with temperature-sensitive mutation of *cdc28*. Overexpression of Ptc2 rendered cells temperature-sensitive on galactose medium, but neither *GAL1*-driven *PTC2* (Fig. 4B) nor the *cdc28-4* genotype (data not shown) was sensitized to *HNT1* status, and thus, a mechanism involving reduced Cdc28 activation by Cak1 in *hnt1* strains is not experimentally supported.

Hnt1 Deficiency Causes Synthetic Loss of Viability with Mutant Kin28-binding Proteins—Given the evidence inconsistent with Hnt1 as a positive regulator of Cak1, we considered the possibility that Hnt1 might be a positive regulator of Kin28. We reasoned that if Hnt1 enzymatic activity is a positive regulator of Kin28, then *hnt1* deletion might cause synthetic loss of viability with destabilization of Ccl1 and Tfb3, the two other TFIID components of general transcription factor TFIID. Precedents for such a prediction are observations that temperature-sensitive mutations in the cyclin H homolog Ccl1 (41) and the MAT1 homolog Tfb3 (42, 64) show synthetic loss of viability, with destabilizing mutations in Kin28. Accordingly, the *hnt1Δ::URA3* deletion was introduced into strains bearing *ccl1-ts4* and *tfb3-rig2ts23* mutations, and plasmid pB42 that expresses *HNT1* was added back to evaluate synthetic loss of viability. As shown in Fig. 4, C and D, growth of *hnt1 ccl1* and *hnt1 tfb3* strains is limited by Hnt1 enzyme activity; both double mutants show synthetic loss of viability that is relieved by yeast or rabbit Hint proteins and not relieved by expression of *hnt1-H116A*. Thus, synthetic interactions with Kin28 and Kin28-interacting proteins Cak1, Ccl1, and Tfb3 suggest that Kin28 function is limited by Hnt1 enzyme activity.

Hnt1 Deficiency Is Synthetically Less Viable with Nonphosphorylatable Kin28—Given that Kin28 is phosphorylated by Cak1 on Thr-162 (33, 34), we reasoned that the basis for Cak1 dependence of *hnt1* deletion strains might be that underphosphorylated Kin28 is hypersensitive to *HNT1* status. The nonphosphorylatable *KIN28-T162A* allele is known to be intragenically synthetically less viable with *kin28-ts16* and to be synthetically less viable with destabilizing mutations in *tfb3* (34). However, prior reports were in apparent disagreement about the phenotype of nonphosphorylatable Kin28 on its own. Although one report presented evidence that *KIN28-T162A* does not complement *kin28-ts3* on galactose (33), another report found no phenotypic consequence of *KIN28-T162A* in experiments performed on glucose (34). Although the authors of the earlier report discovered the existence of two additional mutations that might have been responsible for noncomplementation (65), the apparent conflict might have been explained on the basis that the phenotypic assays differed in carbon source. In fact, although the restrictive temperature for wild-type strains differs in different backgrounds, the *KIN28-T162A* mutation reduces that temperature with respect to isogenic wild-type strains on galactose media. As shown in Fig. 5, nonphosphorylatable Kin28 reduces the restrictive temperature on galactose by ~1–2 °C, whereas *hnt1Δ* reduces the restrictive temperature by 1–2 °C. The effects are additive, such that double mutants cannot grow at temperatures permissive for either single mutant. Thus, synthetic loss of viability on galactose between *KIN28-T162A* and *hnt1Δ* accounts for synthetic loss of viability between *cak1* and *hnt1*. Additionally, it is now possible to reconcile the observations that although cells with a single *KIN28-T162A* mutation are aphenotypic on glucose media (34), they are moderately temperature-sensitive on galactose media (33). Furthermore, in the experiment presented in Fig. 4A, we examined whether the amino acid at the Cak1 phosphorylation site of Kin28 affects the phenotype of *cak1Δ hnt1Δ* or *cak1Δ HNT1* cells. Although *KIN28-T162A* is

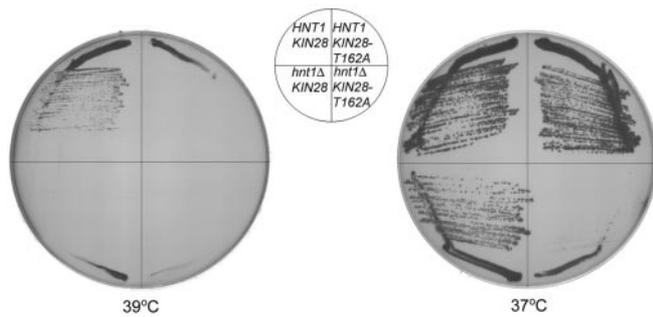


FIG. 5. Nonphosphorylatable Kin28 is limited by Hnt1 for growth on galactose. Strain BY155–8c (genotype *hnt1Δ kin28Δ*, carrying *KIN28* or *KIN28-T162A* on plasmids pB164 and pB165, respectively) was transformed with *HNT1* plasmid pB42 or with empty vector pRS423, and transformants were grown at 39 and 37 °C on SGalC –his –leu media for 6 days.

synthetically less viable with *hnt1Δ* (Fig. 5), the nonphosphorylatable allele is not synthetically less viable with *hnt1Δ* in the absence of Cak1 (Fig. 4A). This confirms that what Cak1 does for Kin28 is simply to phosphorylate it at Thr-162 and that *hnt1Δ* sensitizes cells to hypophosphorylated Kin28.

Kin28 Protein Is Not Conditionally Null in *hnt1* Mutants—Destabilized Tfb3 with nonphosphorylatable Kin28, a genetic combination that is synthetically less viable than either single mutation, leads to Kin28 protein degradation at the nonpermissive temperature (34). Because *hnt1* deletion also is synthetically less viable with *KIN28-T162A*, we tested whether Kin28 might be degraded in *hnt1* mutants on galactose as a function of temperature and discovered that Kin28 is not destabilized by *hnt1* deletion. As shown in Fig. 6A, Kin28 protein has a similar steady state abundance in cells grown at increasing temperatures, and the protein level is not reduced as a function of *hnt1* deletion. Even in *hnt1Δ* cells at 37 °C in galactose media, which are nearing the nonpermissive temperature, Kin28 protein levels are not reduced.

Synthetic loss of viability of *hnt1* with temperature-sensitive alleles of *kin28*, *ccl1*, and *tfb3* suggested Kin28 might be conditionally null in *hnt1Δ* cells or disadvantaged in complex formation. With the observation that Kin28 is not degraded but stable in *hnt1* strains, we tested whether Kin28 overexpression from the *GAL1* promoter is deleterious to *hnt1* mutants. As shown in Fig. 6B, *GAL1*-driven *KIN28* expression mildly inhibits growth of *HNT1* cells and seriously inhibits growth of *hnt1Δ* mutant cells. Thus, a straightforward interpretation of the disadvantage that *hnt1* mutants have, particularly in combination with mutations in TFIIC components or excessive or nonphosphorylated Kin28, is that the Hint/Hnt1 substrate AMP-X inhibits Kin28 complex formation.

DISCUSSION

HIT superfamily proteins are conserved as nucleotide-binding proteins (1). Whereas enzymes in the Fhit and galactose-1-phosphate uridylyltransferase branches are found in subsets of living organisms, Hint orthologs are the ancestral prototypes that contain representatives in archaea, bacteria, and eukarya (19). To this date, no phenotypic consequence of loss of a Hint ortholog alone had been demonstrated in any organism. Loss of Aprataxin, a human Hint-branch protein whose yeast ortholog is Hnt3, is responsible for the progressive neurological disease ataxia with oculomotor apraxia (3, 4), although biochemical substrates or a requirement for enzymatic activity have not been examined. Here we show that in yeast, Hnt1 is conditionally essential for growth on galactose media at elevated temperatures. As was shown earlier, *hnt1* deletion reduces the permissive temperature for *kin28-ts3* cells (20), but this effect

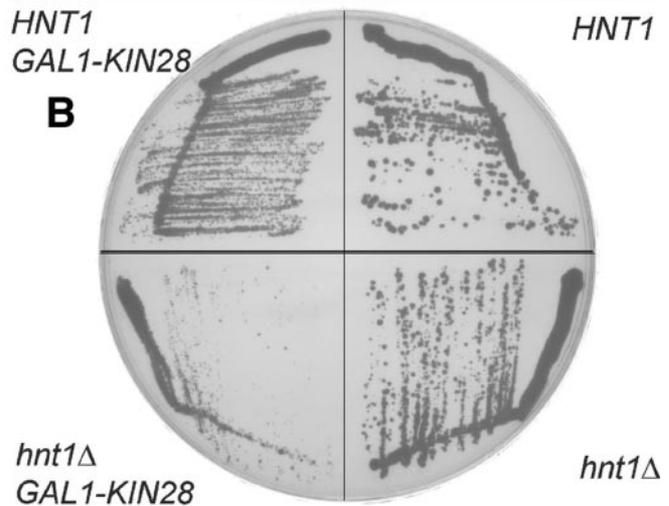
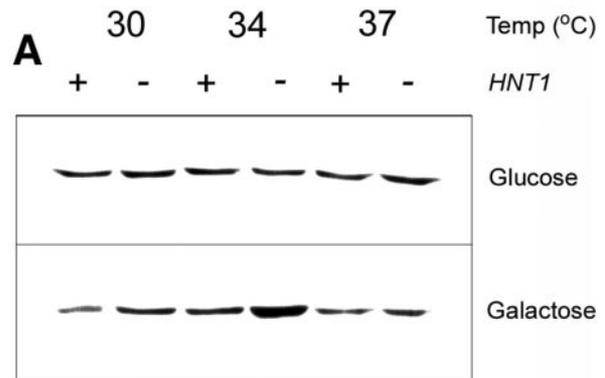


FIG. 6. Kin28 protein is stable in *hnt1* mutants and Kin28 overexpression in *hnt1* mutants inhibits growth on galactose. A, isogenic strains varying only at the *HNT1* locus, expressing HA-tagged Kin28 were grown at the indicated temperatures in yeast extract/peptone/dextrose or yeast/peptone/galactose and subjected to a Western blot with anti-HA antibody. B, strain BY8-5c (genotype *hnt1Δ*) was transformed with *HNT1* plasmid pB42 or empty vector pRS423 and *GAL1-KIN28* plasmid pB194 or the empty pRS425GAL1 control, and transformants were grown at 37 °C on SGalC –his –leu media for 6 days.

is not allele-specific for *kin28*, and synthetic reduction of viability by *hnt1* deletion was discovered with temperature-sensitive mutations in *cak1*, *ccl1*, and *tfb3*, with the nonphosphorylatable *KIN28-T162A* allele, and with *KIN28* overexpression. The single *hnt1Δ* mutant phenotype and the double mutant phenotypes allowed an evaluation of the requirement of the Hnt1 active site. Complete suppression by rabbit Hint protein, which shares only 22% sequence identity with Hnt1, and lack of complementation by Hnt1-His116Ala in every phenotypic assay suggested that Hint homologs have an enzymatic activity responsible for *HNT1* function *in vivo*. We looked for an enzymatic activity in purified Hint and Hnt1 preparations beyond the earlier report of an ADPase activity of $8.5 \text{ M}^{-1} \text{ s}^{-1}$ (54) and discovered that AMPNH₂ is hydrolyzed by these enzymes in an active-site-dependent manner with specificity constants of 2.9×10^6 and $1.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, respectively. Although we do not know whether AMPNH₂ is the authentic *in vivo* substrate of Hnt1, which is provisionally termed AMP-X, we note that AMPNH₂ is found in eukaryotic cells (58) and that the enzyme that produces it from AMP-SO₄, a precursor of all organosulfur compounds, and ammonia has been found in a wide variety of organisms of bacterial and eukaryotic origin (59).

Because of the active-site dependence of Hnt1 function *in*

in vivo and *in vitro* and synthetic loss of viability with *kin28* and *cak1*, we proposed that Hint substrate AMP-X inhibits either Cak1 or Kin28 when *hnt1* is inactivated. Genetic analysis showed that a Cak1 function is made more important in *hnt1*-deficient cells, but this is not due to inhibition of Cak1 because cells that were constructed to live without Cak1 are strongly Hnt1-dependent. Additionally, although *hnt1* deletion attenuates cells to the stability of known Kin28-binding proteins Ccl1 and Tfb3, implicating Kin28 as the direct target of AMP-X, a synthetic phenotype between *hnt1* and *KIN28-T162A* revealed that the Cak1 dependence can be explained by an *hnt1*-inhibited form of Kin28 requiring phosphorylation by Cak1.

Kin28/Cdk7 may not be the only target of Hnt1/Hint regulation. Indeed, in bacteria, failure to cleave AMPNH₂ or a related compound is likely to have completely different consequences because bacteria do not have a Cdk7 homolog. Additionally, it has been appreciated for several years that nucleoside-based prodrugs, such as 2',3'-dideoxy-2',3'-dideohydrothymidine (d4T) can be made more potent by conversion to a 5' phosphoramidate. For example, d4T containing an α -phosphate linked to alanine methyl ester is readily intracellularly available and is converted to the triphosphate after hydrolysis of the nucleoside monophosphoramidate by an unknown intracellular enzymatic activity (66). The apparent identity of Hint to nucleoside monophosphoramidate hydrolase (60) and the fact that Hint and Hnt1 liberate AMP from AMP alanine methyl ester suggest that Hint may be the enzyme responsible for phosphoramidate prodrug maturation *in vivo*.

Accumulation of a Hint/Hnt1 substrate may inhibit Kin28 either by reducing Kin28 function in a way that enforces dependence on Kin28-binding proteins and Cak1 activation or by reducing the ability of Kin28 to form a complex with TFIID components Ccl1 and Tfb3 at elevated temperatures. The stability of Kin28 in *hnt1* deletion strains and the synthetic phenotype of Kin28 overexpression provide some support for Hnt1 as a positive regulator of Kin28 complex assembly and potentially indicate that a form of Kin28 accumulates in *hnt1* cells that has dominant interfering activity, particularly on galactose media. Finally, although the idea that Hnt1 promotes Kin28 function simply by complex formation (20) appears to be incorrect, the two-hybrid interactions reported could be explained if Hnt1 has a protein substrate, namely a nucleotidylated form of Kin28. The possibility that these enzymes have modified proteins as substrates is supported by the observation that Hint and Hnt1 readily hydrolyze a lysyl-AMP derivative. Ongoing work is designed to elucidate the biosynthesis and the identity of AMP-X, other possible targets of Hint and Hnt1 regulation, and to determine whether Aprataxin and Hnt3 function as adenosine monophosphoramidases on either small molecule or macromolecular substrates.

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Supplemental Table 2. Sequences of DNA primers used in this study.

Primer Sequence (5' to 3')

4739	TAAGAAACACATATGGAGCCATTGATATCG
4740	GGAAGCTAACACACCCTCGAGTATAAACTTA
4748	TATGAAGTAAAAGGACAATCAG CACGCCTTCCAGACTTTTAAGAAACATTGACTGAGAGTGCACCA
4749	CTTAAATATGAAAAGAAAACGCTAAAAGAAATCATAGCATGCATTACTCTAGTGCGGTATTTACACCG
4766	CGACCACGTCGCCTTCCATTTG
4787	ACAGGATCCGACGTCTGGAGGCTTCTAC
4795	GACGGTACCATTACTCTAATCGGAGCC
5046	ATGAAAGTGAATATGGAGTACACAAAAGGGTATGTGGGGTTATATATTGTAGATTGACTGAGAGTGCAC
5047	TCAGTTACGTATTTTTATTGAAGACGGGCACCTTGGTGGTGGTAATTCCTCTGTGCGGTATTTACACCG
5048	GACTGGATCCTTGAACAAATCCTCCAAGAG
5049	GACTCTCGAGTCGTCTGCTACTGTTTGTCTC
5052	AGGAATCAAATGGAATGCGACGTGGTCCGACTTC
5054	CATCATATGAAAGTGAATATGGAGTAC
5055	GATCTCGAGTCAGTTACGTATTTTTATTGAAG
5066	CATGGGATCCATTGGATGAACCTACA
5067	CAGTCTCGAGCGAATTCATAATGATGG
5075	TCAGAAAGCACTGGCAAGAACG
5085	TCACGCGGATCCATGGCAGATGAGATTGCC
5086	GATCCGCTCGAGTTAACCAGGGGCCAGTTC
5108	CGACATCGCCGATCCAAAGAAAAATGGGACAAATTCTATCAAACC
5109	CGAAGGATGGAATCCCTTCATTGCAAGTGGGAACGG
5116	TCTTTTCAGCTTCAGCTTCAGCC
5122	ATGAGCGGTGAATTAGCAAATTACAAAAGACTTGAGAAAGTCGGTGAAGGCATAGGCCACTAGTGGATCTG
5123	CTTCATTCGCGCTTCCCAATACTCTGAATATCTTGAAAATCTGATCGATCAGCTGAAGCTTCGTACGC
5124	ATGAAACTGGATAGTATAGACATTACACACTGTCAAGTTGGTCAAATCTACGCATAGGCCACTAGTGGATCTG
5125	CATCCGCAATCTCGTCCAGTCGAGGCATATTCCTCTCAACAATGCTAATCAGCTGAAGCTTCGTACGC
5126	GCCAGCACATCAGCTACAGTG
5127	CCGCGTGGATCTAGACTTGG
5130	CATAGAAGCAAAGAGGCCAGATG
5131	CTTGCAAGATTCTTTGACAGCTCC
5132	ATGAAAGTGAATATGGAGTACACAAAAGGGTATGTGGGGTTATATATTGTGCATAGGCCACTAGTGGATCTG
5133	TCAGTTACGTATTTTTATTGAAGACGGGCACCTTGGTGGTGGTAATTCCTCAGCTGAAGCTTCGTACGC
5134	GCTTCATTTGAAGCATTGTGCAGAG
5135	ATATGTTGAGGATACATCTAATGTC
5190	GACCGCTCGAGTTACTTGTGTCATCGTCTTTGTAGTCGATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCATCGGAGCCTCTAGTTGG
HNT1-3	GACGGTACCATTACTCTAATCGGAGCC
HNT1-5	ACAGGATCCGACGTCTGGAGGCTTCTAC