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

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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 (AMP\(\text{NH}_2\)) 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 TFIIK kinase subcomplex of general transcription factor TFIHII 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)\[\dag\] proteins are a superfamily of nucleotide-binding proteins named for a near C-terminal HAXXHXX motif (X is a hydrophobic amino acid) positioned at the \(\alpha\)-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 hydrolyase 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 galactosesomiasis (1), budding yeast diadenosine tetraphosphate phosphorylases Apal 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 TFIHII 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 TFIHII 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 TFIHII-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
nonsense-enzymatic 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 Hnt28/Cdc11/Tbk3 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 ffb3. 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 Hnt2 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 Hnt1 is only 22% identical to yeast Hnt1, the enzymatic activity of Hnt2 is substantially the same as that of Hnt1, and expression of the rabbit enzyme fully suppressed all hnt1 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 Tbk3 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 HINT1 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 was and a ura+ isolate heterozygous for hnt1 disruption named BY5 were allowed to sporulate, and tetrads were dissected, generating haploid hnt1Δ proge inheritance including BY1-2a, BY3-3b, BY5-4a, and BY5-5c. Additionally, a hnt1::kanMX4 PCR product generated with primers 4748 and 4749 and plasmid pRS400 as template (36) was used to transform haploid wild-type MATa wild-type strain GS812-17c (28) and MATa cdc28-4 strain GP2412 to geneticin resistance to produce hnt1Δ::kanMX4 strain BY158 and hnt1::kanMX4 cdc28-4 strain BY169. Strain BY5-3b was crossed with cdc28-ts2 strain JGV117, cdc28-17a strain JGV110, and cdc28-10a strain JGV112 (38) to generate four strains heterozygous for hnt1 and kin28 alleles named BK2, BK5, BK7, and BK5, respectively, which were dissected to yield a viable diploid with mutant haploid progeny including BK2-2a, BK3-8b, BK7-1c, and BK8-2a.

To introduce Hnt1 into the cdc28-ts164 background, hnt1 strain BY158 was mated with cdc28 strain GP2351 (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 cdc28::HIS3 was obtained by tetrad dissection.

To generate strain BY185 of genotype hnt1Δ::kanMX4 cdc28::HIS3 carrying Cdc28::4324 and alleles of Kin28 on a plasmid, wild-type diploid strain SEY6210.5 (37) was disrupted with a PCR-generated cdc28::loxP-kanMX-::loxP disruption cassette generated by amplification of pGK13::hnt1 and with primers 5122 and 5123. The cdc28::loxP-kanMX-::loxP heterozygous for the cdc28 allele. BY172 was confirmed by amplification with diagnostic primers 5126 and 5127 and then transformed with plasmid pSH47, which expresses cdc28 from the GAL1 promoter (39). A geneticin-sensitive derivative of genotype cdc28::HIS3/CDC28 was cured of plasmid pSH47, named strain BY173, and subjected to transplacement of cdc28::loxP-kanMX-::loxP using plasmids pB159 and pB160. Heterozygosity for the cdc28 allele in strain BY175 was confirmed with primers 5130 and 5131, and the cdc28::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 cdc28::HIS3 derivative was named strain BY178. This strain was transformed with a YCPTRP1 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-fluoroacetic acid-sensitive and contained cdc28::HIS3, cdc28::HIS3, and cdc28::HIS3 disruption alleles, as evidenced by diagnostic PCR. This strain was used to derive the hnt1::kanMX4 disruptant BY185 using primers 4748 and 4749 and cdc28::loxP-kanMX-::loxP primed by diagnostic primers HINT1 and amplified with primers KU22 plasmids pB164, pB165 and pB183, which express KIN28, KIN28, T162A, and KIN28-T162E, respectively, were then transformed into strain BY185 with evi-
digested with BamHI and EcoRI and cloned into BamHI- and EcoRI-cleaved plasmid pRS426GAL1 (46). To create plasmid pB164, containing KIN28 expressed from its own promoter, genomic DNA from SEY26210 was amplified using primers 5086 and 5087. The product was digested with BamHI and EcoRI and cloned into BamHI plus EcoRI-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 BamHI-XhoI fragment of plasmid pB165 was transferred to BamHI- and XhoI-cleaved pRS416 (36). To create plasmid pB193, 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 SpeI and XhoI and inserted into SpeI- plus XhoI-cleaved pRS425GAL1 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 proteinate 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-His116A 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 mM 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-alpha-Alanine methyl ester and AMP-N-epsilon(N-acetyl lysine methyl ester), which were synthesized according to a modification of the synthesis of AMP-N-alpha-Lysine (49), and human DNA ligase I adenylated intermediate, a gift of Alan E. Tomkinson, which was prepared as described (50). Compounds were screened for hydrolysis in 10-ul reactions containing 1 mM substrate, 0.5–2.0 ug of enzyme, 0.5 mM MgCl2, 20 mM Na-PiPES at pH 7.2 for 5–12 h at 37°C. Reactions repeated without MgCl2 were within 10% of the values reported. Reactions were started at 100 ul 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 absorbance 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 colorometrically at 400 nm as described (50). Human DNA ligase I adenylated intermediate (50 pmol) was treated with 1 u of enzyme in 50 ul 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°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 u⁻¹ were assayed further in 50–200-ul reactions with 0.2 mM substrate, 0.005–1 u of enzyme, and 20 mM PIPES, pH 7.2, for 10 min at 37°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 ul with 0.5–3 ng of enzyme. In some cases, 10 mM Na-BeS or 10 mMimidazole HCl at pH 7.2 was used as buffer because of an interfering peak in PIPES. The low Kₘ 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 Kₘ of 68 nm should be considered an upper limit.

**Evaluation of Hint and Kin28 Abundance in Vivo**—To examine steady state abundance of FLAG-tagged wild-type and active-site mutant proteins, strain BY8-5c was transformed with empty vector pRS415 and with pRS413, which encodes for FLAG-tagged Hnt1 constructs. Transforms were generated at 37°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. 1X Complete protease inhibitor mixture...
 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 fmol min⁻¹ µg⁻¹) but not yeast Hnt1. Purine nucleosides 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-APAp, diadenosine polyphosphates ApppA through ApppppppA, 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 AMPoS indicated that sulfate and sulfur are much better leaving groups than phosphate for substrate hydrolysis. Moreover, discovery of AMPNH₂ as a substrate allowed the demonstration that Hint and Hnt1 are more than 100,000-fold less proficient on this substrate (34) than on ADP (54).

In a focused search for adenosine mononucleotides with a leaving group other than phosphate, we found that AMP morpholidate, a nucleotide with 5'-phosphoramide (P-N) linkage to a six-membered ring, was cleaved 200-fold better than ADP by yeast Hnt1, 2.9 × 10⁶ M⁻¹ s⁻¹ and 1.0 × 10⁶ M⁻¹ s⁻¹, respectively. No AMP was detected (Table I). Because 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 (kcat/Km for rabbit and yeast, 2.9 × 10⁶ M⁻¹ s⁻¹ and 1.0 × 10⁶ M⁻¹ s⁻¹, respectively) than on ADP (54).

A review of the literature shows that AMPNH₂ is found in eukaryotic cells (58), is synthesized from AMPoS 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),

### Table I

| Substrate                        | Rabbit Hint | Yeast Hnt1 | Hnt1-H116A |
|----------------------------------|-------------|------------|------------|
| AMP-NH₂ (200 µM)                 | 1.71 ± 0.25 (3) | 7.57 ± 0.97 (4) | <.00003 (4) |
| AMP-N-[N'-a-acetyl lysine methyl ester] (200 µM) | 1.03 ± 0.13 (4) | 5.92 ± 1.05 (4) |           |
| AMP-N-alanine methyl ester (200 µM) | 0.79 ± 0.11 (4) | 2.70 ± 0.33 (3) |           |
| AMP morpholidate (200 µM)        | 0.82 ± 0.15 (3) | 1.37 ± 0.49 (3) |           |
| AMP(SO₄) (200 µM)                | 0.15 ± 0.03 (4) | 0.020 ± 0.004 (3) |           |
| AMPS(O₄) (200 µM)                | 0.075 ± 0.011 (5) | 0.029 ± 0.005 (4) |           |
| ADP                              | 0.0039 ± 0.0012 (2) | 0.00054 ± 0.00007 (2) |           |
| GDP                              | 0.0028 ± 0.0005 (4) | 0.00017 ± 0.000006 (3) |           |
| ADP-ribose                       | 0.0012 ± 0.0002 (2) | 0.00042 ± 0.00026 (2) |           |
| 8-Br-ADP                          | 0.00090 ± 0.0003 (2) | <0.00012 (2) |           |
| NAD                              | 0.00090 ± 0.0003 (2) | <0.00012 (2) |           |
| AppA                             | 0.00046 ± 0.0001 (2) | <0.00012 (2) |           |
| IDP                              | 0.00035 (1) | <0.00011 (1) |           |
| NADH                             | 0.00022 ± 0.0001 (2) | <0.00001 (2) |           |
| ATP                              | 0.00013 ± 0.00002 (2) | <0.00012 (2) |           |
| DNA ligase 1 intermediate (1 µM)  | <0.00002 (1) | <0.00001 (1) |           |
| p-Nitrophenyl TMP (100 µM)       | <0.001 (2) | <0.001 (2) |           |
| ApppAppA, ApppppA, AppppppA, ApppppA, d(pApA), acetyl-CoA, GTP, GDP-glucose, ITP, UTP, UDP, UDP-glucose, CTP, CDP, 8-Br-ATP | <0.00011 (2) | <0.00012 (2) |           |

**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(▲).
Hnt1 Enzymatic Activity Promotes Kin28 Function

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-α-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 AMPNHz, 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 hnt1Δ 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 hnt1Δ 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 hnt1Δ double mutants, single mutants are slightly enlarged with respect to wild-type cells, 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 hnt1Δ kin28-ts8 strain BKS–2a with plasmids expressing wild-type HNT1 (pB42) or no insert (pRS423). B, an hnt1Δ kin28-ts8 strain (BKS–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 hnt1Δ cak1-civ1ts4 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.

To test whether mutation of Cak1, which is not only the CAK for Kin28 (27–29) but also the CAK for Kin28 (33, 34), also increases the cellular requirement for Hnt1, a strain bearing the cak1-ts Civ1ts4 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 AMPNHz, 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 discovered the cellular reasons why mutation of the other kinase also enforces a requirement for Hnt1.
**Hnt1 Enzyme Activity Promotes Kin28 Function**

**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 Cc1 and Tfb3, the two other TFIIF components of general transcription factor TFIIH. Precedents for such a prediction are observations that temperature-sensitive mutations in the cyclin H homolog Cc1 (41) and the MAT1 homolog Tfb3 (42, 64) show synthetic loss of viability, with destabilizing mutations in Kin28. Accordingly, the hnt1::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 tft3 strains is limited by Hnt1 enzyme activity; both double mutants show synthetic loss of viability that is relieved by yeast or rabbit Hnt1 proteins and not relieved by expression of hnt1-H116A. Thus, synthetic interactions with Kin28 and Kin28-interacting proteins Cak1, Ccl1, and Tfb3 suggest that Hnt1 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 tft3 (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 isoegenic 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 hnt1Δ cells. Although KIN28-T162A is
its growth of $HNT1$ mutant cells. Thus, a straightforward interpretation of the AMP-X inhibits Kin28 complex formation. nonphosphorylated Kin28, is that the Hint/Hnt1 substrate stabilization with mutations in TFIIK components or excessive or $GAL1$ shown in Fig. 6B, from the stable in mutation. With the observation that Kin28 is not degraded but hnt1 conditionally null 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 $hnt1\Delta$) 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 $hnt1\Delta$ 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 AMPNH$_2$ is hydrolyzed by these enzymes in an active-site-dependent manner with specificity constants of $2.9 \times 10^6$ and $1.0 \times 10^5 \text{M}^{-1}\text{s}^{-1}$, respectively. Although we do not know whether AMPNH$_2$ is the authentic in vivo substrate of Hnt1, which is provisionally termed AMP-X, we note that AMPNH$_2$ is found in eukaryotic cells (58) and that the enzyme that produces it from AMPSo$_4$, 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 synthetically less viable with $hnt1\Delta$ (Fig. 5), the nonphosphorylatable allele is not synthetically less viable with $hnt1\Delta$ 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 $hnt1\Delta$ 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 also 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 $hnt1\Delta$ 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 $hnt1\Delta$ cells or disadvantaged in complex formation. With the observation that Kin28 is not degraded but stable in $hnt1$, 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 $hnt1$ mutant cells. Thus, a straightforward interpretation of the disadvantage that $hnt1$ mutants have, particularly in combination with mutations in TFIIK 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 prototypical 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...
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 hint1 is inactivated. Genetic analysis showed that a Cak1 function is made more important in hint1-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 hint1 deletion attenuates cells to the stability of known Kin28-binding proteins Ccl1 and Tbh3, implicating Kin28 as the direct target of AMP-X, a synthetic phenotype between hint1 and Tbh3 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 AMPPNP, 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 compound is likely to have completely different consequences and monophosphoramide by an unknown intracellular enzymatic 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 hydrolyase (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 moduration 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 TFIK components Ccl1 and Tbh3 at elevated temperatures. The stability of Kin28 in hint1 deletion strains and the synthetic phenotype of Kin28 overexpression provide some support for Hint1 as a positive regulator of Kin28 function simply by complex formation (20) appears to be incorrect, the two-hybrid interactions reported could be explained if Hint1 has a protein substrate, namely a nucleotidylation 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 Apratuxan and Hnt3 function as adenosine phosphomonomidases on either small molecule or macromolecular substrates.

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