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

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Abstract

The histidine 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 GalT-related transferases. While 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 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 temperature. 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 TFIIH, 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.

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Histidine triad (HIT)[1] proteins are a superfamily of nucleotide-binding proteins named for a near C-terminal HisϕHisϕHisϕϕ motif (ϕ, 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 (GalT), which is the enzyme deficient in galactosemics (1), budding yeast diadenosine tetraphosphate phosphorylases Apa1 and Apa2 (17), and adenylylsulfate:phosphate adenylyltransferase (18). Ironically, though 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 general transcription factor TFIIH 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 (CTDK), is required at post-initiation steps in TFIIH function (26). In yeast, CAK and CTDK activities are separated not only by virtue of different requirements for regulatory subunits but also by virtue of different catalytic subunits. In *S. cerevisiae*, the CAK for Cdc28 is encoded by monomeric Cak1 (27-29) while the major CTDK of yeast is Kin28, which is TFIIH-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 CTDK, 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. Further, we were interested in determining whether an enzymatic or a 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

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[1]The abbreviations used are

HIT
: histidine triad

GalT
: galactose-1-phosphate uridylyltransferase

CAK
: cyclin-dependent kinase activating kinase

CTDK
: RNA polymerase II carboxy-terminal domain kinase

AMP-NH_2
: adenosine-5′-monophosphoramidate.

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TFIIH, i.e., the yeast Kin28-Ccl1-Tfb3 complex and the mammalian Cdk7-cyclin H-MAT1 complex, as TFIIK (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 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 adenosine-5′-monophosphoramidate ($AMPNH_2$) to AMP plus a presumptive ammonia leaving group and that the active site histidine we show biochemically to be required for $AMPNH_2$ 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 TFIIK 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 while 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 2, which is available as a supplement) 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 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 *MAT*α wild-type strain GF312-17c (28) and *MATa 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.

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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-Thr162Ala*, 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-Thr162Ala*, and *KIN28-Thr162Glu*, 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-Thr162Ala*, 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

E. 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 BamHI and KpnI, cloned into BamHI and KpnI-digested pRS423 (44) to generate plasmid pB42. Plasmid pB150 was generated by site-directed mutagenesis (45) of pB4 using primer

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5052 to generate the *hnt1-His116Ala* 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 BamHI and XhoI, and cloned into BamH1 and XhoI-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 BamHI and XhoI-cleaved product was cloned into BamHI and XhoI-cleaved pRS413 (36). To create plasmid pB217 containing the triple FLAG-tagged *hnt1-His116Ala* 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 NdeI and XhoI and cloned into NdeI and XhoI-cleaved pSGA02. To create plasmid pB195 containing *HNT1-His116Ala* 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 BamHI and EcoRI and cloned into BamHI and EcoRI-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 BamHI and EcoRI, cloned into BamHI plus EcoRI-cleaved plasmid pRS415 (36). Site directed mutagenesis of plasmid pB164 using primer 5075 generated plasmid pB165 containing *KIN28-Thr162Ala*. Mutagenesis of pB164 using primer 5116 generated pB183 containing *KIN28-Thr162Glu*. To construct plasmid pB192, the BamHI-XhoI fragment of plasmid pB165 was transferred to BamHI and XhoI-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 SpeI and XhoI and inserted into SpeI plus XhoI-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-His116Ala*) according to published procedure (2). Frozen and thawed cell pellets (250 mg wet weight) were lysed in 12.5 ml buffer A (20 mM Tris pH 7.5, 150 mM NaCl) with one EDTA-free protease inhibitor cocktail tablet (Roche). Lysates were cleared by centrifugation and further cleared after precipitation with 1 mg 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 μM adenosine for Hint and 10 mM adenosine for Hnt1-His116Ala. 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 α-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

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were screened for hydrolysis in 10 μl reactions containing 1 mM substrate, 0.5-2.0 μg enzyme, 0.5 mM $MgCl_2$, 20 mM Na PIPES at pH 7.2 for 5-12 hr at 37 °C. Reactions repeated without $MgCl_2$ were within 10% of the values reported. Reactions were diluted to 100 μl and analyzed by liquid chromatography on a MonoQ HR 5/5 column (Pharmacia) with mobile phases that were suitable mixtures of water and 1.25 M NH_4 HCO_3, pH 8. Ultraviolet absorbing peaks corresponding to nucleoside monophosphate products were integrated and converted to nmole 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 μg of enzyme in 50 μl of ligase buffer (30 mM Tris HCl, 50 mM NaCl, 0.5 mM DTT, 1 mM EDTA, 10% glycerol, pH 7.5) for 1 hr 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} μg^{-1} were assayed further in 50-200 μl reactions with 0.2 mM substrate, 0.005-1 μg 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 μl with 0.5-3 ng 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 °C in SGalC -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 glass beads (0.5 mm, Sigma) in 1 ml of cold 150 mM Tris acetate, pH 7.5, 300 mM $(NH_4)_2SO_4$, 1 mM spermidine, 1 mM dithiothreitol, 10% glycerol, 1x Complete protease inhibitor cocktail (Roche) with 7 one-minute pulses of a Mini-8 Beadbeater (Biospec). Twenty μg of total protein (determined by Biorad 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), washed, and developed with ECL reagents (Amersham) as instructed. To detect HA-tagged Kin28 protein, strains YGL26 and BY186 were grown to an optical density (600 nm) of 0.6 in YPGal and YPD medium at 30 °C, 34 °C and 37 °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 °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 *hnt1Δ::TRP1* and *hnt1Δ::URA3* and introduced them into diploid strains. Diploids heterozygous for *hnt1Δ* 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 *hnt1Δ* mutants in comparison with isogenic *HNT1* isolates revealed that *hnt1Δ* mutants do

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not grow on rich or synthetic galactose-containing media at 39 °C (Fig. 1A). Transformation of *hnt1Δ* mutants with a plasmid carrying an intact *HNT1* gene restored full growth on galactose at 39 °C, but empty vector did not. As shown in Fig. 1B, *hnt1Δ* 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 GalT (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 ($k_{cat}/K_m = 8.5\ M^{-1}\ s^{-1}$) (54). Discovery of a physiological assay for yeast Hnt1 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 1, under the conditions of our assay, which could detect as little as 30 fmol of nucleoside monophosphate released per minute per microgram of enzyme, ATP was detectable as a weak substrate of rabbit Hint (130 fmol $min^{-1}\ \mu g^{-1}$) 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 acetylCoA 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 $AMPSO_4$ and AMPαS indicated that sulfate and sulfur are much better leaving groups than phosphate for Hint as these compounds were cleaved 20 to 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 nmol $min^{-1}\ \mu g^{-1}$. 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 two-fold better than AMP morpholidate. The smallest possible amine substitution of AMP, $AMPNH_2$, was the best substrate, being consumed at 1.7 and 7.6 nmol $min^{-1}\ \mu g^{-1}$ by rabbit and yeast enzymes, respectively.

We performed initial rate determinations as a function of the concentration of $AMPNH_2$. As shown in Fig. 2, $AMPNH_2$ is hydrolyzed to AMP plus a presumptive ammonia leaving group with k_{cat} and K_m values of $0.20 \pm 0.05\ s^{-1}$ and 68 ± 15 nM (n=3) for the rabbit enzyme and values of $0.87 \pm 0.25\ s^{-1}$ and 870 ± 150 nM (n=3) for the yeast enzyme. To test whether these activities depend on the conserved middle histidine of the HIT motif, which is required for catalytic activity of Fhit (13,55-57), a His116Ala mutant form of yeast Hnt1 was generated, purified from *E. coli*, and incubated with the same substrates. No AMP was detected (Table 1). As the HPLC assay detects enzyme activity at levels 100,000-fold lower than those observed from enzymatic hydrolysis of $AMPNH_2$, active-site dependence was shown conclusively. Moreover, discovery of $AMPNH_2$ as a substrate allowed the demonstration that Hint and Hnt1 are over 100,000-fold more proficient on this substrate (k_{cat}/K_m for rabbit and yeast, $2.9 \times 10^6\ M^{-1}\ s^{-1}$ and $1.0 \times 10^6\ M^{-1}\ s^{-1}$, respectively) than on ADP (54).

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Review of the literature shows that $AMPNH_2$ is found in eukaryotic cells (58), is synthesized from $AMPSO_4$ 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 1. 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-αacetyl lysine methyl ester) (Table 1). 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 Hnt1are postulated to function *in vivo* by hydrolyzing $AMPNH_2$ 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 temperature, the *His116Ala* 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-His116Ala* showed no complementation, demonstrating that the active site histidine residue is necessary for physiological function of the protein. Though the yeast Hnt1-His116Ala 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-His116Ala* was caused by protein instability rather than loss of enzymatic activity. We therefore constructed C-terminally triple FLAG-tagged (47) *HNT1* and *hnt1-His116Ala* 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 while FLAG-tagged *hnt1-His116Ala* did not (not shown). Having validated the constructs, we probed soluble lysates for presence of the FLAG-tagged antigens at 37 °C, which is the highest temperature at which the FLAG-tagged *hnt1-His116Ala* strain can be grown. As shown in Fig. 1C, the *hnt1-His116Ala* 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 while double mutants are highly elongated and cease

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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 BK8-2a with plasmids expressing wild-type *HNT1*, *hnt1-His116Ala*, rabbit *HINT*, or with an empty vector. As shown in Fig. 3B, yeast Hnt1 and rabbit Hint proteins were fully functional while 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-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 $AMPNH_2$, 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.

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 GlyXGlyXXGly sequence that nearly all protein kinases use to bind ATP and is uniquely *resistant* to 5′-fluorosulfonylbenzoyladenosine (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 tested whether *hnt1Δ* produces 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

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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 TFIIK components of general transcription factor TFIIH. 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. 4C and 4D, 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-His116Ala*. 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 Thr162 (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-Thr162Ala* 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. While one report presented evidence that *KIN28-Thr162Ala* does not complement *kin28-ts3* on galactose (33), another report found no phenotypic consequence of *KIN28-Thr162Ala* in experiments performed on glucose (34). Though the authors of earlier report discovered 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, while the restrictive temperature for wild-type strains differs in different backgrounds, the *KIN28-Thr162Ala* mutation reduces that temperature with respect to isogenic wild-type strains. As shown in Fig. 5, nonphosphorylatable Kin28 reduces the restrictive temperature on galactose by ~1 to 2 °C while *hnt1Δ* reduces the restrictive temperature by 1 to 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-Thr162Ala* and *hnt1Δ* accounts for synthetic loss of viability between *cak1* and *hnt1*. Additionally, it is now possible to reconcile the observations that while cells with a single *KIN28-Thr162Ala* mutation are aphenotypic on glucose media (34), they are moderately temperature-sensitive on galactose media (33). Further, 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. While *KIN28-Thr162Ala* is 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 Thr162 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). As *hnt1* deletion also is also synthetically less viable with *KIN28-Thr162Ala*, 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.

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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 *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/Hnt1substrate AMP-X inhibits Kin28 complex formation.

DISCUSSION

HIT superfamily proteins are conserved as nucleotide-binding proteins (1). Whereas enzymes in the Fhit and GalT 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) though 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 temperature. As was shown earlier, *hnt1* deletion reduces the permissive temperature for *kin28-ts3* cells (20), but this effect 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-Thr162Ala* 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 M^{-1} s^{-1} (54), and discovered that $AMPNH_2$ is hydrolyzed by these enzymes in an active site-dependent manner with specificity constants of 2.9×10^6 M^{-1} s^{-1} and 1.0×10^6 M^{-1} s^{-1} , respectively. Though 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 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, while *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-Thr162Ala* 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 $AMPNH_2$ or a related compound is likely to have completely different consequences as 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′-didehydro-thymidine can be made more potent by conversion to a 5′ phosphoramide. For example, 2′,3′-dideoxy-2′,3′-didehydro-

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thymidine containing an α-phosphate linked to alanine methylester is readily intracellularly available and is converted to the triphosphate after hydrolysis of the nucleoside monophosphoramide 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 phosphoramide 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 TFIIK 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, while 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 whether Aprataxin and Hnt3 function as adenosine monophosphoramidases on either small molecule or macromolecular substrates.

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REFERENCES

1. Brenner C, Garrison P, Gilmour J, Peisach D, Ringe D, Petsko GA, Lowenstein JM. Nat. Struct. Biol 1997;4:231–238. [PubMed: 9164465]
2. Gilmour J, Liang N, Lowenstein JM. Biochemical Journal 1997;326:471–7. [PubMed: 9291120]
3. Date H, Onodera O, Tanaka H, Iwabuchi K, Uekawa K, Igarashi S, Koike R, Hiroi T, Yuasa T, Awaya Y, Sakai T, Takahashi T, Nagatomo H, Sekijima Y, Kawachi I, Takiyama Y, Nishizawa M, Fukuhara N, Saito K, Sugano S, Tsuji S. Nat Genet 2001;29:184–8. [PubMed: 11586299]
4. Moreira MC, Barbot C, Tachi N, Kozuka N, Uchida E, Gibson T, Mendonca P, Costa M, Barros J, Yanagisawa T, Watanabe M, Ikeda Y, Aoki M, Nagata T, Coutinho P, Sequeiros J, Koenig M. Nat Genet 2001;29:189–93. [PubMed: 11586300]
5. Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Druck T, Croce CM, Huebner K. Cell 1996;84:587–597. [PubMed: 8598045]
6. Siprashvili Z, Sozzi G, Barnes LD, McCue P, Robinson AK, Eryomin V, Sard L, Tagliabue E, Greco A, Fusetti L, Schwartz G, Pierotti MA, Croce CM, Huebner K. Proc. Natl. Acad. Sci. USA 1997;94:13771–13776. [PubMed: 9391102]
7. Ji L, Fang B, Yeh N, Fong K, Minna JD, Roth JA. Cancer Res 1999;59:3333–3339. [PubMed: 10416589]
8. Sard L, Accornero P, Tornielli S, Delia D, Bunone G, Campiglio M, Colombo MP, Gramegna M, Croce CM, Pierotti MA, Sozzi G. Proc. Natl. Acad. Sci. USA 1999;96:8489–8492. [PubMed: 10411902]
9. Dumon KR, Ishii H, Vecchione A, Trapasso F, Baldassarre G, Chakrani F, Druck T, Rosato EF, Williams NN, Baffa R, During MJ, Huebner K, Croce CM. Cancer Res 2001;61:4827–4836. [PubMed: 11406559]

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10. Fong LYY, Fidanza V, Zanesi N, Lock LF, Siracusa LD, Mancini R, Siprashvili Z, Ottey M, Martin SE, Dolsky R, Druck T, McCue PA, Croce CM, Huebner K. Proc. Natl. Acad. Sci. USA 2000;97:4742–4747. [PubMed: 10758156]
11. Dumon KR, Ishii H, Fong LYY, Zanesi N, Fidanza V, Mancini R, Vecchione A, Baffa R, Trapasso F, During MJ, Huebner K, Croce CM. Proc. Natl. Acad. Sci. USA 2001;98:3346–3351. [PubMed: 11248081]
12. Huang Y, Garrison PN, Barnes LD. Biochem. J 1995;312:925–932. [PubMed: 8554540]
13. Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, Croce CM, Ohta M, Huebner K. Biochem 1996;35:11529–11535. [PubMed: 8794732]
14. Pekarsky Y, Campiglio M, Siprashvili Z, Druck T, Sedkov Y, Tillib S, Draganescu A, Wermuth P, Rothman JH, Huebner K, Buchberg AM, Mazo A, Brenner C, Croce CM. Proc. Natl. Acad. Sci. USA 1998;95:8744–8749. [PubMed: 9671749]
15. Pace HC, Hodawadekar SC, Draganescu A, Huang J, Bieganowski P, Pekarsky Y, Croce CM, Brenner C. Curr Biol 2000;10:907–17. [PubMed: 10959838]
16. Draganescu A, Hodawadekar SC, Gee KR, Brenner C. J. Biol. Chem 2000;275:4555–4560. [PubMed: 10671479]
17. Huebner K, Garrison PN, Barnes LD, Croce CM. Ann. Rev. Genet 1998;32:7–31. [PubMed: 9928473]
18. Bruser T, Selmer T, Dahl C. Journal of Biological Chemistry 2000;275(3):1691–8. [PubMed: 10636864]
19. Brenner C, Bieganowski P, Pace HC, Huebner K. J. Cell. Physiol 1999;181:179–187. [PubMed: 10497298]
20. Korsisaari N, Makela TP. J. Biol. Chem 2000;275:34837–34840. [PubMed: 10958787]
21. Fisher RP, Morgan DO. Cell 1994;78:713–24. [PubMed: 8069918]
22. Fisher RP, Jin P, Chamberlin HM, Morgan DO. Cell 1995;83:47–57. [PubMed: 7553872]
23. Devault A, Martinez AM, Fesquet D, Labbe JC, Morin N, Tassan JP, Nigg EA, Cavadore JC, Doree M. Embo Journal 1995;14:5027–36. [PubMed: 7588631]
24. Serizawa H, Makela TP, Conaway JW, Conaway RC, Weinberg RA, Young RA. Nature 1995;374:280–2. [PubMed: 7885450]
25. Shiekhattar R, Mermelstein F, Fisher RP, Drapkin R, Dynlacht B, Wessling HC, Morgan DO, Reinberg D. Nature 1995;374:283–7. [PubMed: 7533895]
26. Akoulitchev S, Makela TP, Weinberg RA, Reinberg D. Nature 1995;377:557–60. [PubMed: 7566158]
27. Kaldis P, Sutton A, Solomon MJ. Cell 1996;86:553–64. [PubMed: 8752210]
28. Thuret JY, Valay JG, Faye G, Mann C. Cell 1996;86:565–76. [PubMed: 8752211]
29. Espinoza FH, Farrell A, Erdjument_Bromage H, Tempst P, Morgan DO. Science 1996;273:1714–7. [PubMed: 8781234]
30. Feaver WJ, Svejstrup JQ, Henry NL, Kornberg RD. Cell 1994;79:1103–9. [PubMed: 8001136]
31. Valay JG, Simon M, Dubois MF, Bensaude O, Facca C, Faye G. Journal of Molecular Biology 1995;249:535–44. [PubMed: 7783209]
32. Cismowski MJ, Laff GM, Solomon MJ, Reed SI. Molecular and Cellular Biology 1995;15:2983–92. [PubMed: 7760796]
33. Espinoza FH, Farrell A, Nourse JL, Chamberlin HM, Gileadi O, Morgan DO. Molecular and Cellular Biology 1998;18:6365–73. [PubMed: 9774652]
34. Kimmelman J, Kaldis P, Hengartner CJ, Laff GM, Koh SS, Young RA, Solomon MJ. Molecular and Cellular Biology 1999;19:4774–87. [PubMed: 10373527]
35. Kaiser, C.; Michaelis, S.; Mitchell, A. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press; Plainview, NY: 1994.
36. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD. Yeast 1998;14:115–32. [PubMed: 9483801]
37. Robinson JS, Klionsky DJ, Banta LM, Emr SD. Mol. Cell. Biol 1988;8:4936–4948. [PubMed: 3062374]
38. Valay JG, Simon M, Faye G. Journal of Molecular Biology 1993;234:307–10. [PubMed: 8230216]

39. Guldener U, Heck S, Fielder T, Beinhauer J, Hegemann JH. Nucleic Acids Research 1996;24:2519–24. [PubMed: 8692690]
40. Cross FR, Levine K. Molecular and Cellular Biology 1998;18:2923–31. [PubMed: 9566911]
41. Valay JG, Dubois MF, Bensaude O, Faye G. Comptes Rendus de L Academie Des Sciences. Serie Iii, Sciences de La Vie 1996;319:183–9.
42. Faye G, Simon M, Valay JG, Fesquet D, Facca C. Molecular and General Genetics 1997;255:460–6. [PubMed: 9294030]
43. Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. Short Protocols in Molecular Biology. 3rd. Wiley; New York: 1995.
44. Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P. Gene 1992;110:119–122. [PubMed: 1544568]
45. Kunkel TA, Roberts JD, Zakour RA. Methods in Enzymology 1987;154:367–382. [PubMed: 3323813]
46. Mumberg D, Muller R, Funk M. Nucleic Acids Research 1994;22:5767–8. [PubMed: 7838736]
47. Herren B, Pech M. J Recept Res 1993;13:725–738. [PubMed: 8450508]
48. Ghosh S, Lowenstein JM. Gene 1997;176:249–255. [PubMed: 8918261]
49. Rossomando EF, Hadjimichael J. International Journal of Biochemistry 1986;18:481–4. [PubMed: 3011538]
50. Tomkinson A, Totty N, Ginsburg M, Lindahl T. Proc. Natl. Acad. Sci. USA 1991;88:400–404. [PubMed: 1988940]
51. Razzell WE. Methods in Enzymology 1963;6:236–258.
52. Lee H-J, Wilson IB. Biochem. Biophys. Acta 1971;242:519–522. [PubMed: 5161034]
53. Wedekind JE, Frey PA, Rayment I. Biochemistry 1996;35:11560–11569. [PubMed: 8794735]
54. Lima CD, Klein MG, Hendrickson WA. Science 1997;278:286–90. [PubMed: 9323207]
55. Lima CD, Damico KL, Naday I, Rosenbaum G, Westbrook EM, Hendrickson WA. Structure 1997;5:763–774. [PubMed: 9261067]
56. Pace HC, Garrison PN, Robinson AK, Barnes LD, Draganescu A, Rosler A, Blackburn GM, Siprashvili Z, Croce CM, Huebner K, Brenner C. Proc. Natl. Acad. Sci. USA 1998;95:5484–9. [PubMed: 9576908]
57. Abend A, Garrison PN, Barnes LD, Frey PA. Biochem 1999;38:3668–3676. [PubMed: 10090754]
58. Fankhauser H, Berkowitz GA, Schiff JA. Biochemical and Biophysical Research Communications 1981;101:524–32. [PubMed: 7306093]
59. Fankhauser H, Schiff JA, Garber LJ. Biochemical Journal 1981;195:545–60. [PubMed: 6274307]
60. Kuba M, Okizaki T, Ohmori H, Kumon A. Int J Biochem 1994;26:235–45. [PubMed: 8174758]
61. Gumport RI, Lehman IR. Proc. Natl. Acad. Sci. USA 1971;68:2559–63. [PubMed: 4944632]
62. Enke DA, Kaldis P, Holmes JK, Solomon MJ. Journal of Biological Chemistry 1999;274:1949–56. [PubMed: 9890950]
63. Cheng A, Ross KE, Kaldis P, Solomon MJ. Genes and Development 1999;13:2946–57. [PubMed: 10580002]
64. Feaver WJ, Henry NL, Wang Z, Wu X, Svejstrup JQ, Bushnell DA, Friedberg EC, Kornberg RD. Journal of Biological Chemistry 1997;272:19319–27. [PubMed: 9235928]
65. Espinoza FH, Farrell A, Nourse JL, Chamberlin HM, Gileadi O, Morgan DO. Molecular and Cellular Biology 2000;20:1898.
66. Balzarini J, Karlsson A, Aquaro S, Perno C-F, Cahard D, Naesens L, DeClercq E, McGuigan C. Proc Natl Acad Sci USA 1996;93:7295–7299. [PubMed: 8692986]

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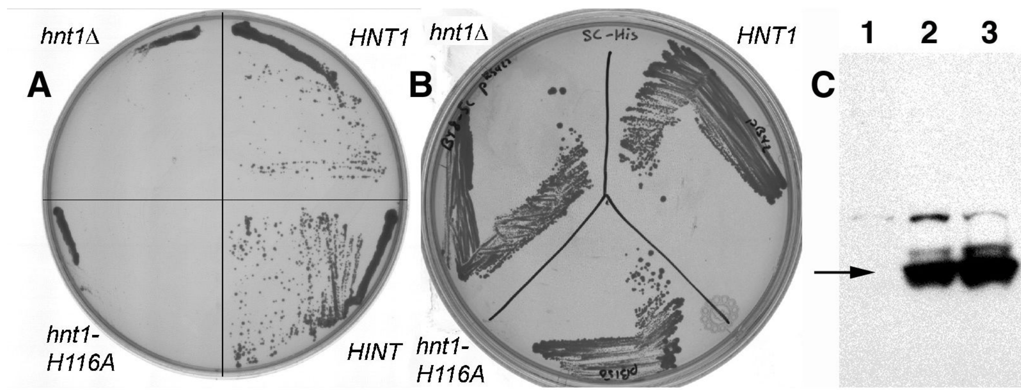

Fig. 1. Hnt1 enzyme activity is necessary for growth on galactose at 39 °C and complemented by rabbit Hint

(A) An *hnt1Δ* strain (BY8-5c) was transformed with plasmids containing either *HNT1* (pB42), *hnt1-His116Ala* (pB150), rabbit *HINT* cDNA under *GAL1* control (pB159), or an empty vector (pRS423), and colonies were grown at 39 °C on SGalRafC media for six days.

(B) Strain BY8-5c transformants containing either *HNT1* (pB42), *hnt1-His116Ala* (pB150), or empty vector (pRS423) were grown at 39 °C on SDC media for four 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 SGalC -his at 37 °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.

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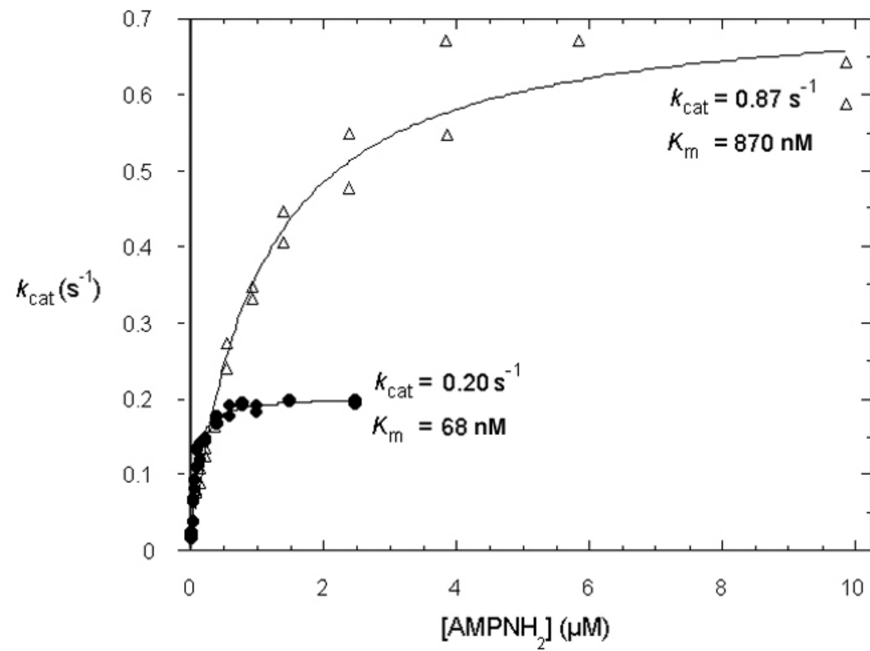

Fig. 2. Hint proteins are adenosine monophosphoramidases

Substrate-concentration dependent rates (per monomer active site) for $AMPNH_2$ hydrolysis to AMP plus the presumptive ammonia leaving group by rabbit Hint (•) and yeast Hnt1(Δ).

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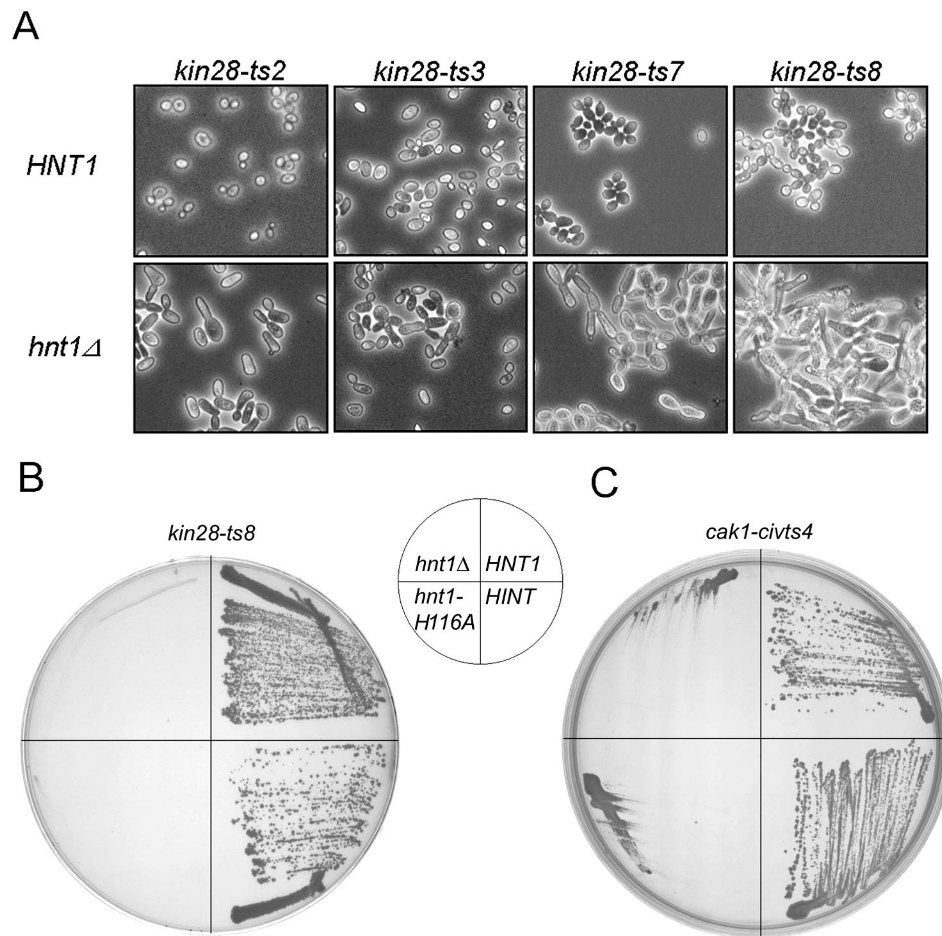

Fig. 3. Synthetic loss of viability between *hnt1Δ* and temperature sensitive alleles of *kin28* and *cak1*

(A) Micrographs of strains carrying an *hnt1* deletion with the indicated temperature-sensitive alleles of *kin28* grown in SGalRafC -leu at 30 to 32 °C, bearing multicopy plasmids containing either wild-type *HNT1* (pB42) or no insert (pRS423).

(B) An *hnt1Δ kin28-ts8* strain (BK8-2a) was transformed with plasmids containing either *HNT1* (pB42), rabbit *HINT* cDNA under *GAL1* control (pB159), *hnt1-His116Ala* (pB150), or an empty vector (pRS423), and grown at 34 °C on SGalRafC media for six days.

(C) An *hnt1Δ cak1-civ1ts4* strain (BY164) was transformed with plasmids containing either *HNT1* (pB42), rabbit *HINT* cDNA under *GAL1* control (pB159), *hnt1-His116Ala* (pB150), or an empty vector (pRS423), and grown at 34 °C on SGalRafC media for six days.

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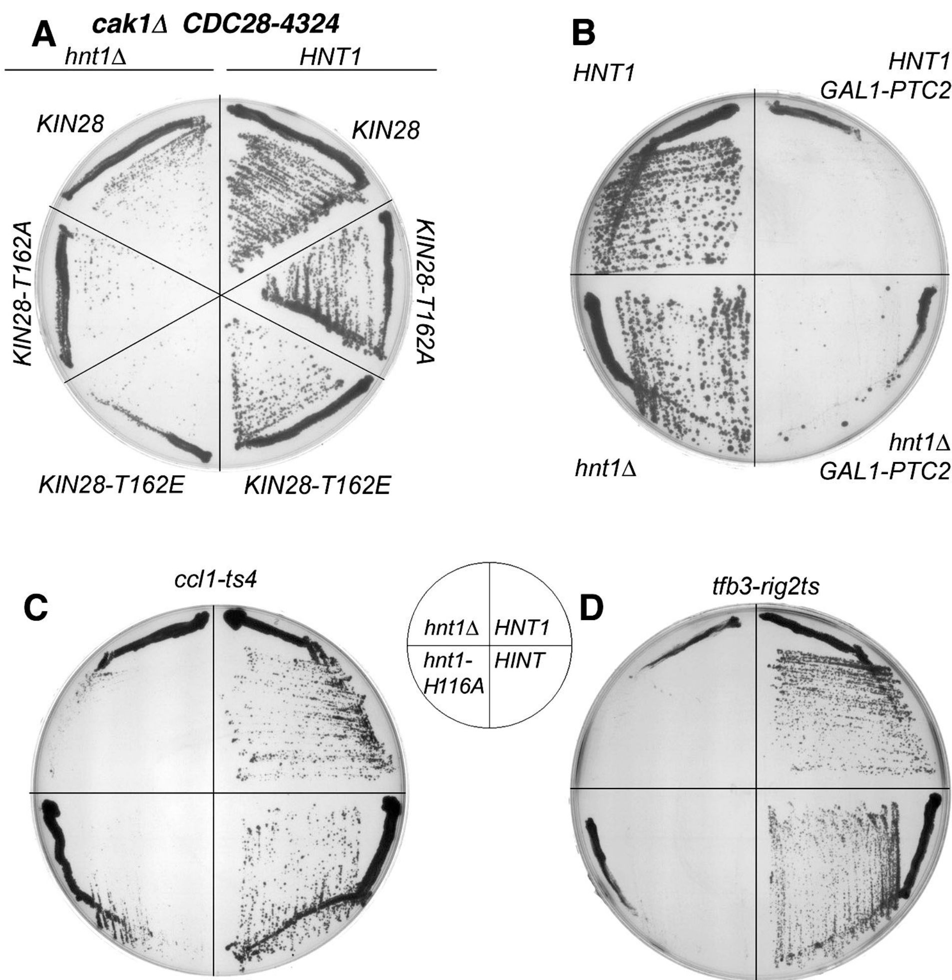

Fig. 4. Cak1 is not the target of Hnt1 regulation and strains with destabilized Kin28-binding proteins are limited By Hnt1 enzyme activity

(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-His116Ala* (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-His116Ala* (pB150), or an empty vector (pRS423), and grown at 34 °C on SGalRafC media for 6 days.

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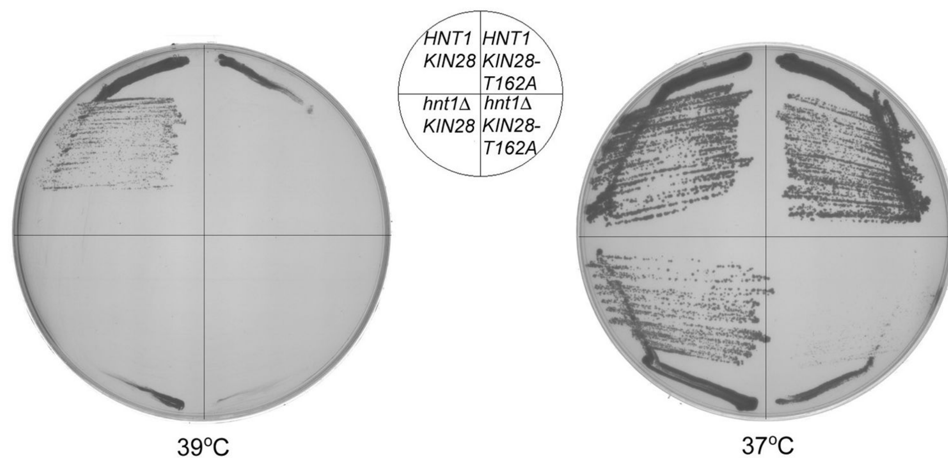

Fig. 5. Nonphosphorylatable Kin28 is limited by Hnt1 for growth on galactose

Strain BY155-8c (genotype *hnt1Δ kin28Δ*, carrying *KIN28* or *KIN28-Thr162Ala* on plasmids pB164 and pB165, respectively) was transformed with *HNT1* plasmid pB42 or with empty vector pRS423, and transformants were grown at 39 °C and 37 °C on SgalC -his -leu media for 6 days.

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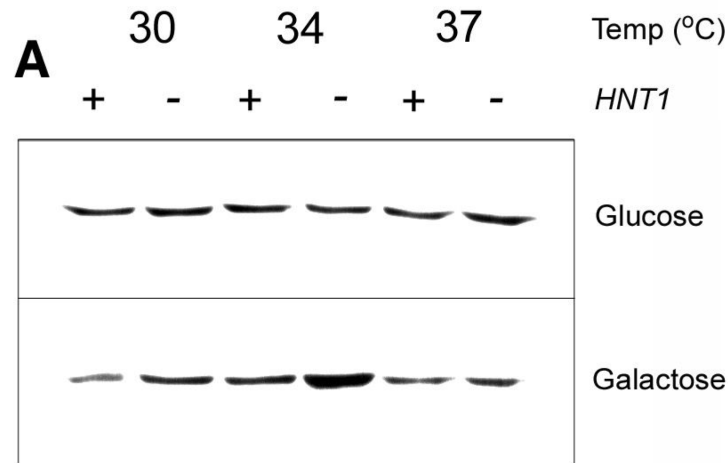

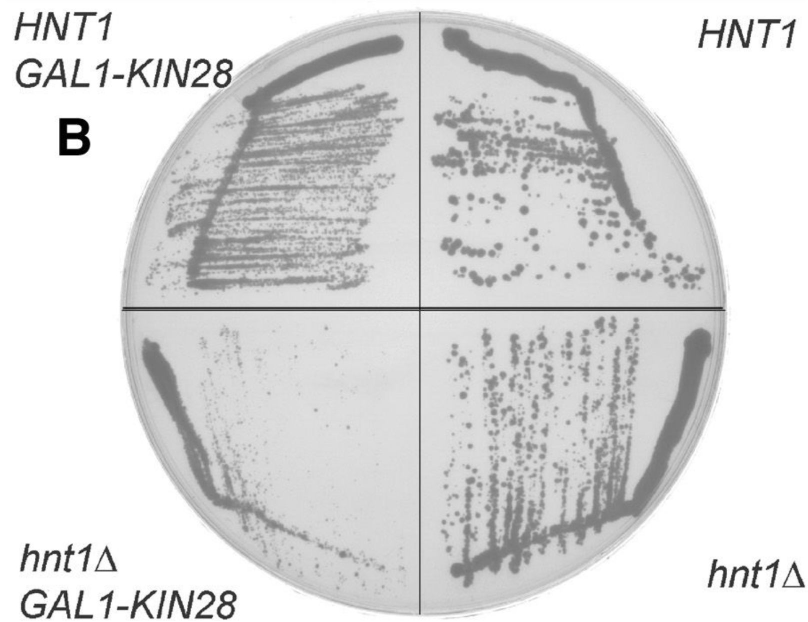

Fig. 6. Kin28 protein is stable in *hnt1* mutants and Kin28 overexpression in *hnt1* mutants inhibits growth on galactose

(A) Isogenic strains varying only at the *HNT1* locus, expressing HA-tagged Kin28 were grown at the indicated temperatures in YDP or YPGal 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 pRS425*GAL1* control and transformants were grown at 37 °C on SGalC -his -leu media for 6 days.