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Research article

The yeast *ISN1* (*YOR155c*) gene encodes a new type of IMP-specific 5'-nucleotidase

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Abstract

Background: The purine salvage enzyme inosine 5'-monophosphate (IMP)-specific 5'-nucleotidase catalyzes degradation of IMP to inosine. Although this enzymatic activity has been purified and characterized in *Saccharomyces cerevisiae*, the gene encoding IMP 5'-nucleotidase had not been identified.

Results: Mass spectrometry analysis of several peptides of this enzyme purified from yeast allowed identification of the corresponding gene as *YOR155c*, an open reading frame of unknown function, renamed *ISN1*. The deduced Isn1p sequence was clearly not homologous to 5'-nucleotidases from other species. However, significant similarities to Isn1p were found in proteins of unknown function from *Neurospora crassa*, *Plasmodium falciparum* and several yeast species. Knock-out of *ISN1* resulted in the total loss of IMP-specific 5'-nucleotidase activity, thus confirming that the *ISN1* gene indeed encodes the enzymatic activity purified from yeast. *In vivo* studies revealed that, when IMP is overproduced through constitutive activation of the IMP *de novo* synthesis pathway, *ISN1* is required for excretion of inosine and hypoxanthine in the medium.

Conclusion: We have identified a new yeast gene, *ISN1* (*YOR155c*), as encoding IMP-specific 5'-nucleotidase activity. The *ISN1* gene defines a new type of 5'-nucleotidase which was demonstrated to be functional *in vivo*.

Background

Purine salvage pathway allows interconversion of bases, nucleosides and nucleotides. Importance of this pathway in humans has been clearly established since several defects in purine salvage enzymes have been associated to pathologies such as mental retardation and severe immunodeficiencies [1,2]. In yeast, mutations in several purine salvage genes lead to deregulation of AMP biosynthesis [3] and purine excretion. Taking advantage of complete genome sequencing of *Saccharomyces cerevisiae* we were able to identify several new purine salvage genes such as adenosine kinase or purine nucleoside phosphorylase [4,5] which are highly homologous to their mammalian counterpart. Strikingly, no 5'-nucleotidase encoding gene could be inferred from comparison of the completely sequenced yeast genome with 5'-nucleotidase genes from

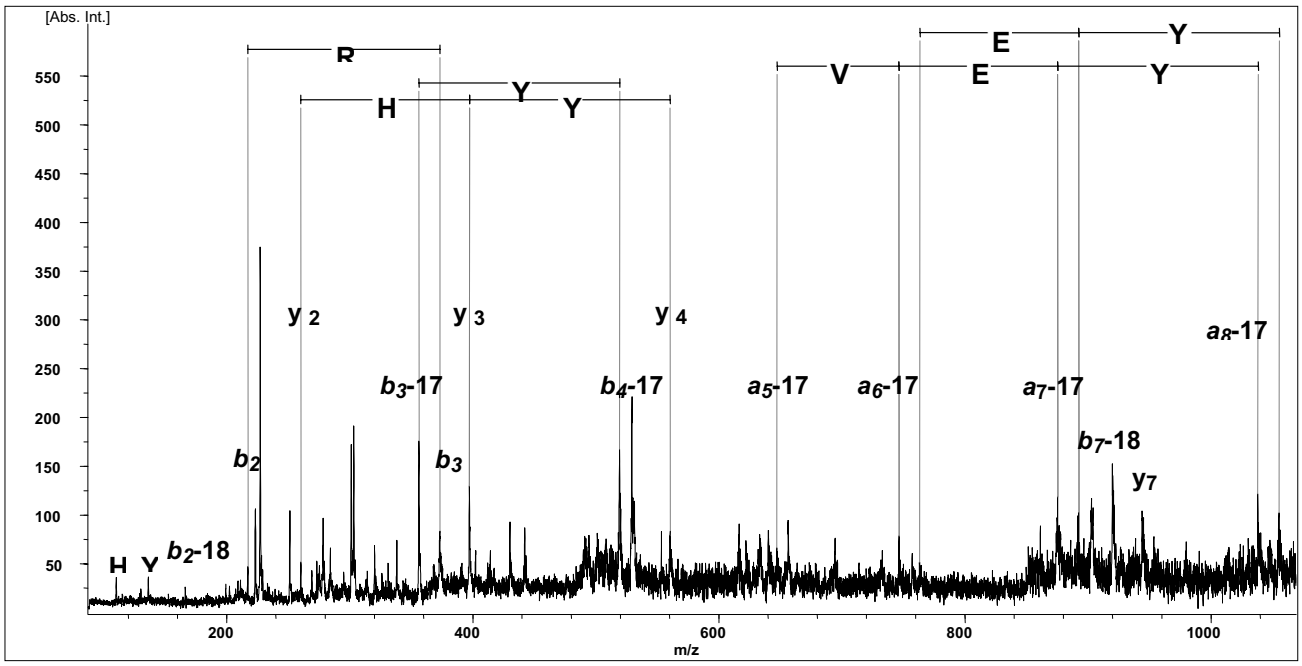

Figure 1

Partial spectrum of PSD fragment ions obtained from m/z 1479.76 precursor ion. Masses of N-terminal ion series of the b and a type [12] are in agreement with the modified sequence Acetyl-SSRYRVEYHK. His and Tyr immonium ions, as well as C-terminal ions (y type) confirm the sequence of the modified peptide.

other species, although several lines of evidence argued for the presence of 5'-nucleotidase in yeast. First, an IMP-specific 5'-nucleotidase activity was detected and purified from yeast [6]. Second, deregulation of IMP biosynthesis resulted in increased production of IMP which was excreted in the medium as a mix of inosine and hypoxanthine [7], thus establishing that, *in vivo*, IMP could be efficiently degraded to inosine in yeast.

In this paper, we report the identification of the yeast IMP-specific 5'-Nucleotidase (*ISN1*) gene encoding a new type of 5'-nucleotidase.

Results and discussion

IMP-specific 5'-nucleotidase corresponds to Yor155p

The IMP-specific 5'-nucleotidase purified as described previously [6] was submitted to EndoLysC proteolytic treatment. The MALDI-MS spectrum of the peptide mixture generated by EndoLysC proteolysis revealed 9 major peaks in the m/z 1000–3000 range, that were used for data base search (Table 1). YOR155c, an open reading frame of unknown function (Swissprot/TrEMBL accession number Q99312) was identified with 8 peptide matches within an 0.1 Da error range (MS-Fit search, see Experimental). Identification was further confirmed by an independent data base search (MS-Tag) with PSD fragment ions obtained from a precursor ion measured at 1845.91 Da (calculated mass 1845.98 Da, matching the 318–334 stretch of the protein, Table 1). The unmatched peptide ion, measured at 1479.73 Da, was submitted to PSD analysis (Figure 1) and assigned to the N-terminal peptide SSRYRVEYHLK having undergone methionine excision and bearing an acetyl blocking group (calculated mass of the modified peptide 1479.77 Da). Based on mass spectrometry identification and further results presented below, YOR155c was renamed ISN1 (IMP-specific 5'-Nucleotidase).

ISN1 *encodes an atypical 5'-nucleotidase*

Search for conserved motif in *S. cerevisiae* Isn1p revealed a typical leucine zipper sequence (Fig. 2) that could be involved in multimerization of the protein which was first isolated as a tetramer [6]. Indeed, Yor155p was found to interact with itself in a systematic two hybrid analysis [13]. The sequence analysis of the *ISN1* gene product revealed that it does not have significant homologies with any 5'-nucleotidase from other organisms neither eucaryotes or prokaryotes nor to the *S. cerevisiae* pyrimi-

Table 1: Assignment of major peptide ions observed in the MALDI-ToF mass spectrum of the EndoLysC protein digest Mass values are monoisotopic for [M+H]+ ions. For the N-terminal peptide, the calculated mass takes into account a modification by methionine excision (- 131.04 Da) and acylation (+ 42.01 Da), the theoretical mass of the unmodified peptide being 1568.80 Da.

Measured mass (Da)	Calculated mass (Da)	Stretch location	Sequence ions identified by PSD analysis
1051.48	1051.51	17–24	
1076.49	1076.53	282–290	
1201.67	1201.64	273–281	
1479.76	1479.77	2–12	y_2-y_4, b_2-b_8, a_5-a_8, H and Y immonium ions
1538.92	1538.94	305–317	
1559.78	1559.80	13–24	
1732.96	1732.98	291–304	
1792.97	1792.98	224–240	
1845.99	1845.98	318–334	y_2-y_4, b_2-b_5, a_4-a_7, R,V and Y immonium ions

```
Sc ---MSSRYRVEYHLKSHRKDEFIDWVKGLLASPFVLHAVSH-----EGDYN-DDLATT-QRVRSQYADIFK--DIEGLIKDKIEFDSRNMSQDEIEDGAS
Pf MKNLDINTFDNIEDIPLGSSEQDPYDFFTLSDRNVMNSDMKKNIVQWNSRYSYNQLKNKDSLIMFLVEIFRSLFVSNCIDKNIDN-----VLLSIEEMFI
Sp ---MASRYRVEYALKQHRKDDFIEWIKGLLSVPFVLQAG-------TPSQKLVTREKE-QETLERYAVILR--DVEKLIEAHIQI---------VSEGGS
Nc ---MASLPPSCWMFLPARLAESTEWIKALLAVPFVLYSQPHG-VLEDPDRSVDTLSQTREEAHRRYSEIFR--DIEAMIDDHIAH------QNDAENPFP
                              *    *                                  *         *   *

Sc SQSLNILGQSRLNLLVPSIGTFFTELPLEQAFLWEDSQRAISARRMVAPSFNDIRHILNTAQIFHFKKQENLHNGKVLRLVTFDGDVTLYEDGGSLVYTN
Pf DHYYN-PQHSRLKYLIDDVGIFFTKLPITKAFHTYNKKYRITKRLYAPPTFNEVRHILNLAQILSLEEG--------LDLLTFDADETLYPDGHDFN--D
Sp --------YSQLKMLVPSITIFWTPLPLVKAMYALEPKLCLAKRSFVAPSFNDVRAILGGAQLNYLADNG-------LDMVSFDGDVTLYEDGQPLLPDN
Nc ---------SKLKLLVPSIGPFFTRLPLEAAFKFQDNKRYISSRRFVSPSFNDIRLILNSAQIMAVTTYG------TLQLATFDGDVTLYDDGQSLEPTS
            * *  *      * * **   *           *    * **  * **  **               *    ** * *** **

Sc PVIPYILKLLRCGINVGIVTAAGYD--EAGTYENRLKGLIVALHDSTDIPVSQKQNLTIMGGESSYLFRYYEDPEEDNFGFRQIDKEEWLLPRMKAWSLE
Pf EVLASYISCLLKKMNIAIVTAASYNN-DAEKYQKRLENLLKYFSKH-NIKDGSYKNFYVMGGESNYLFKCNEEAT-----LYSVPENEWR-HYKKFVDYD
Sp PVISRLIQLLSRGIYVIILTAAGYPSRTGQEYTDRFSGLLQAIEDS-DLKDGQKRKLHVLGGESNYLFAYDP-----LHGLQWVDADSWMLPVMKTWPHD
Nc PVIPRLLDLLRKNVKIGIVTAAGYT--TADRYYSRLHGLLDAMANSADLTPSQKQSLVVMGGEANYLFEFDSSSP---HLLAPVPRQHWLTPEMAAWNEQ
    *       *       * *** *       *  *   *                      ***  ***                    *

Sc DVEKTLDFAERTLNRLRKRLNLPSEISIIRKVRAVGIVPGER-YDEASKRQVPVKLDREQLEEIVLTLQNTLES--FAPSRRIQFSCFDGGSDVWCDIGG
Pf TVQEILNISEKCLEKVIKDFGLC--AQIQRKEKSIGLVPNKIPSLNIKNEQKNYMIKYEVLEEAVIRIKKEIIK----NKITAPYCAFNGGQDLWVDVGN
Sp EILEILDTAEETLRSCIQGLNID--AKIVRKERSVGFVP------SLG-----QKLRREQLEEAVLETQATLQI----RNFSVPFTAFNGGNDIWCDIGD
Nc DIAQLLDVAEAALRDCIKTLNLP--ATLMRKDRAVGIIP-------VSP---EIRIPRESLEETVLLVQKILELSTVGRSRRVPFCAFNGGRDVFVDIGD
        *   *  *               **    *  *                  * *** *                      * ** *   * *

Sc KDLGVRSLQQFYN----PESPIQPSETLHVGDQFAPVGSANDFKARLAG-----CTLWIASPQETVNYLHRLLETD-------------
Pf KAEGLLILQKLLK--------IQKKKCCHIGDQFLHSG--NDFPTRLDLYIFCSLTLWVSNPQETKACLKSIMHLNIKSFIPEVLYENQ
Sp KKLGVLCLQHFFN-------ISHPSRCLHVGDQFLSAG-NNDYKARAAA-----TTVWVSSPHETIEFLDYYFSFLKK-----------
Nc KSWGVTVCQRWFSQKEGPHGVIKGENTLHVGDQFLSAG-ANDFRARSVG-----TTAWIASPVETVELLDELAELMGKKMS--------
   *  *    *                  * ****   *  **   *         * *   * **   *
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Figure 2

Sequence comparison of Isn1p and putative proteins from various species. The amino acid sequence deduced from the nucleotide sequence of the *YOR155c* ORF (Isn1p) from *S. cerevisiae* (Sc) was compared with putative proteins derived from complete genome sequencing of *Plasmidium falciparum* (Pf) *Schizosaccharomyces pombe* (Sp) and *Neurospora crassa* (Nc). Accession numbers are presented in Table 2. Residues conserved in all four proteins are shown by asterisks. Dashes indicate the gaps created for alignment. The putative leucine zipper sequence in Isn1p is highlighted in grey.

dine 5'-nucleotidase encoded by the *SDT1* gene [14]. The only significant similarities were found with proteins of unknown function derived from complete genome sequencing of *Schizosaccharomyces pombe, Neurospora crassa* and *Plasmodium falciparum* (Fig. 2) as well as partially sequenced open reading frames of *Saccharomyces bayanus*, *Saccharomyces exiguus*, *Kluyveromyces lactis*, *Pichia angusta*, *Pichia sorbitophila* (Table 2).

Table 2: Sequence comparison between Isn1p and similar proteins from various species.

Species	Identity %	Length of alignment	Accession number
Plasmodium falciparum	33	349	AAN36150
Neurospora crassa	44	440	EAA34993
Schizosaccharomyces pombe	44	448	CAB10798
Pichia angusta	50	328	(a)
Pichia sorbitophila	51	251	(a)
Kluyveromyces lactis	66	112	(a)
Saccharomyces exiguus	83	24	(a)
Saccharomyces bayanus	94	196	(a)
Saccharomyces cerevisiae	100	450	CAA99361

(a) genolevure program http://cbi.labri.u-bordeaux.fr/Genolevures/

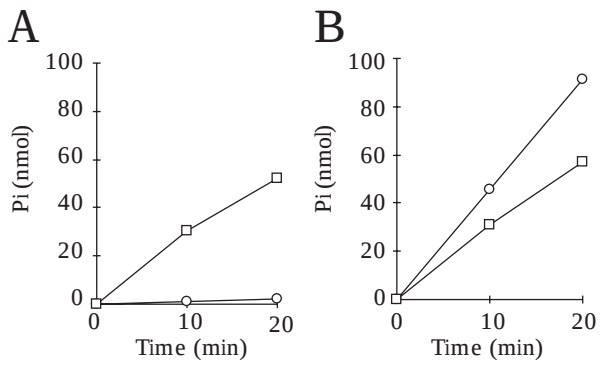

Figure 3
IMP 5'-nucleotidase activity is undetectable in an *isn1* mutant. Time course of hydrolysis of IMP (circles) or phenylphosphate (squares) in crude extracts from the *isn1* knock-out strain (A) or wild-type strain (B). Reaction mixtures contained 10 mM IMP or phenylphosphate, 25 mM $MgCl_2$, and 0.1% BSA in 100 mM imidazole/HCl (pH 6.5). The amounts of protein in the cell extracts used in these experiments were 270 μg for both *isn1* knock-out and wild type strains.

It therefore seems that IMP degradation in these species occurs through an atypical enzyme. Strikingly, the other yeast IMP metabolizing enzymes such as IMP dehydrogenase and adenylosuccinate synthetase are highly similar to bacterial and mammalian enzymes [15,16]. Interestingly, hypoxanthine-guanine phosphoribosyl transferase from yeast is also poorly conserved [3] except among yeast species, while other purine salvage enzymes such as adenosine kinase or purine nucleoside phosphorylase are highly conserved from yeast to mammals [4,5]. It therefore clearly appears that some purine salvage enzymes are derived from a common ancestor with mammalian enzymes, while others have evolved differently in yeast and mammals. This intriguing observation could be useful for future development of specific antifungal drugs.

Activities of 5'-nucleotidase and non-specific phosphatase in yeast cell extracts

To evaluate the role of Isn1p in IMP-specific 5'-nucleotidase activity, the enzymatic activity was measured in extracts from a wild-type strain and an isogenic *isn1* knock-out mutant. IMP-hydrolyzing activity in the extract from wild-type strain was ca 17×10^{-3} units/mg protein, while the activity in the extract from the mutant strain was virtually undetectable (Fig. 3). The hydrolyzing-activities with phenylphosphate, which is not a substrate for IMP 5'-nucleotidase [6], were ca 12×10^{-3} units/mg for the knock-out strain and ca 11×10^{-3} units/mg for the wild-type strain (Fig. 3.). Similar results were obtained in experiments assessing phosphomonoesterase activities with various phosphomonoesters as substrates (Table 3). IMP-hydrolyzing activity in the crude extracts from the *isn1* knock-out strain was lower than 3% of that in the wild-type strain. Activities with each tested phosphomonoester other than IMP were almost equal in both strains.

These results clearly show that in the absence of the *ISN1* gene, the IMP-specific 5'-nucleotidase activity is absent. Together with identification of the purified protein by mass spectrometry, this result clearly establishes that *ISN1* encodes yeast IMP-specific 5'-nucleotidase.

Role of Isn1p in vivo

Because of its high substrate specificity, Isn1p most probably regulates intracellular IMP amounts. To test the role of Isn1p *in vivo*, we took advantage of mutants in the *ADE4* gene increasing the flux in the IMP biosynthesis pathway [7]. In these mutants, hypoxanthine and inosine are excreted in the growth medium, most probably as a

Table 3: Specific activities in cell extracts with various phosphomonoesters Each reaction mixture contained 10 mM substrate, 25 mM $MgCl_2$, 0.1% BSA in 100 mM imidazole/HCl (pH 6.5)

Substrates	Activity (unit × 10^3/mg protein)	
	Knock-out strain	Wild-type strain
IMP	0.4	16.6
AMP	1.2	0.7
GMP	0.6	0.5
UMP	0.5	0.4
CMP	0.4	0.3
Phenylphosphate	11.7	11.2
1-Glycerophosphate	106.4	98.8

result of degradation of overproduced IMP [7]. We therefore used such an IMP overproducing mutant to assay whether IMP degradation would be affected in an *isn1* mutant strain. A plasmid (P2047) overexpressing a dominant mutant form of *ADE4* leading to purine excretion [7] was transformed in wild-type and *isn1* yeast strains and purine excretion was assayed. Indeed, crossfeeding experiments revealed that hypoxanthine excretion (allowing growth of a halo of hypoxanthine auxotrophic cells) was much lower in an *isn1* mutant strain (Fig. 4A). Analysis of the purine compounds excreted in the growth medium by isogenic wild-type and *isn1* mutant strains revealed very different excretion patterns (Fig. 4B). While the P2047-transformed wild-type strain excreted inosine, hypoxanthine and also a significant amount of IMP (Fig. 4B) (compared to the non-excreting control, not shown), in the P2047-transformed mutant strain, IMP only was still efficiently excreted, whereas neither inosine nor hypoxanthine were detected in the growth medium (Fig. 4B). This result clearly shows that Isn1p plays a critical role in IMP degradation *in vivo*.

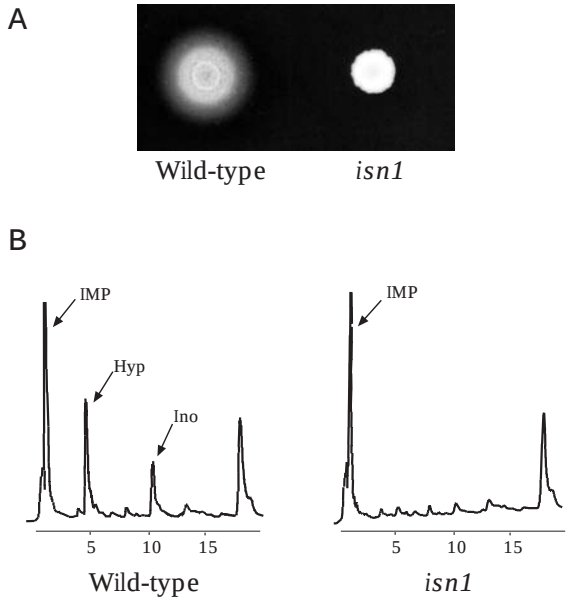

Figure 4
Excretion of purine compounds in the *isn1* mutant strain. (A.) Purine excretion phenotype of the wild-type strain (BY4741) and the *isn1* mutant strain transformed with the P2047 plasmid leading to IMP overproduction. Transformed strains were spotted on a lawn of *ade1* homozygous diploid cells plated on purine-free SD casa medium. Purine excretion was monitored after 3 days at 30°C. (B.) HPLC analysis of excreted purine compounds. Transformed strains as in (A) were grown in adenine- and uracil-free SD casa medium. Cells were then harvested and the medium was filtered. Separation of purine compounds was achieved by HPLC and monitored by following absorbance at 260 nm. Specific peaks were identified as IMP, hypoxanthine and inosine as described previously [7]. Arrows indicate the identified peaks with the following abbreviations: Hyp, hypoxanthine; Ino, inosine.

Why should yeast have a specific IMP 5'-nucleotidase? One possibility is that Isn1p could be involved in scavenging IMP toxic derivatives or analogs. Our attempts to document such an effect were unsuccessful : the *isn1* knockout mutant, grown in the presence of several purine analogs (8-azaadenine, 6-chloropurine, 8-azaguanine, 6-mercaptopurine, 2,6-diaminopurine...), did not show any growth alteration compared to the isogenic wild-type strain (data not shown). Alternatively, Isn1p could play some role in DNA repair. Indeed, transcriptome analysis revealed that *ISN1* transcription increased three folds when cells were treated with methyl methanesulfonate, a DNA damaging agent [17], and furthermore, Isn1p co-purified with Mlh1p [18], a protein involved in mismatch repair [19]. It is therefore tempting to propose that Isn1p could for example be involved in removing dIMP residues resulting from deamination of dAMP. However, while the mutagenic effect of dIMP has been documented [20], this deoxynucleotide is a poor substrate for Isn1p *in vitro* [6]. Whatever its involvement in DNA damage response, it is clear that Isn1p activity is present in normally growing cells [6]. This result together with the extreme substrate specificity of Isn1p argues for a physiological role of Isn1p in IMP metabolism. Deregulation of IMP production leads to excretion of inosine and hypoxanthine [7], suggesting that IMP should not normally accumulate, and we have shown (Fig. 4) that Isn1p is clearly required for this process. However, overproduction of IMP in an *isn1* mutant strain did not result in any obvious growth pheno-

type that would help us to further understand why yeast cells have developped such an efficient way of degrading IMP to inosine and hypoxanthine. Therefore, while the enzymatic activity of Isn1p is well characterized, its physiological role still remains unclear.

Conclusions

In this paper we report the identification of a new yeast gene, *ISN1* (*YOR155c*), as encoding IMP-specific 5'-nucleotidase activity. The *ISN1* gene is the first member of a new type of 5'-nucleotidase which appears conserved among several species, from yeast to human malaria parasite. IMP-specific 5'-nucleotidase was found to be functional *in vivo* although no clear physiological role could be associated to Isn1p activity. Beside future physiological work, identification of Isn1p will make possible structural analysis required to further characterize this new type of nucleotidase.

Methods

Purification of IMP-specific 5'-nucleotidase for amino acid sequence analyses

Yeast IMP-specific 5'-nucleotidase was purified as described previously [6] and run on a 15% one-dimensional SDS-PAGE [8]. Fixation and staining of proteins in the gel were achieved by a two hours treatment with 0.003% Amido Black in 45% ethanol, 10% acetic acid. The gel was then washed several times with water to remove staining excess and the band of interest was cut out and washed in a large volume of water to remove acetic acid and ethanol.

In-Gel Protein Digestion

The purified 5'-nucleotidase protein band was washed three times with 250 µl of 50% acetonitrile in 25 mM Tris-HCl (pH 8.6) for 30 min at room temperature with gentle agitation. The band was then sliced into small pieces (~1 × 1 × 0.75 mm) and partially dried under vacuum in a SpeedVac concentrator. The gel was rehydrated in digestion buffer (0.1 M Tris-HCl, pH 8.6, 10% acetonitrile) containing 5 µg/mL of endoproteinase Lys-C (Roche Applied Science). Digestion was carried out for 16 h at 37°C in a minimal volume allowing total immersion of gel pieces and was stopped by addition of 1% (final concentration) aqueous trifluoroacetic acid (TFA). Peptides recovered from the digestion mix and from the gel pieces through three cycles of extraction with 60% acetonitrile, 0.1%TFA in water, were then pooled and concentrated in a Speed-Vac. Finally, a micro-chromatographic separation step of proteolytic digest was performed by means of a C18 ZipTip (Millipore), using a 10 µl sample load. The elution was achieved with 2 µl of 70% acetonitrile, 0.1% TFA in water

Mass spectrometry

The matrix assisted laser desorption-ionization (MALDI) mass spectrometer was a Reflex III (Bruker) instrument equipped with a 337 nm laser source, ion gate and ion reflector suitable for Post Source Decay (PSD) experiments. α-Cyano-4-hydroxy-cinnamic acid (Sigma) was used as a matrix (saturated solution in 50% acetonitrile, 0.1% aqueous TFA). The dried droplet method was used for sample loading on stainless steel targets. External mass calibration was achieved with a mixture of eight peptides (angiotensin, bradikynin, bombesin, ACTH clips and oxidized insulin B chain, all from Sigma) covering the m/z 1000–3500 range. For PSD analysis, the reflector voltage was stepped down in 10 to 12 steps, starting from 30 kV, in order to collect fragment ions from the precursor down to immonium ions. Data base search was effected by means of the Protein Prospector MS-Fit ans MS-Tag search engines http://falcon.ludwig.ucl.ac.uk, using a mass tolerance of 0.2 Da for precursor ions, and 0.5 Da for PSD fragment ions.

Yeast strains and media

Yeast strains used in this study were purchased from Euroscarf.

BY4741: *MAT***a** *ura3Δ0 leu2Δ0 met15Δ0 his3Δ1*

Y02411: *MAT***a** *ura3Δ0 leu2Δ0 met15Δ0 his3Δ yor155c::KanMX4*

SD is 0.17% nitrogen base, 2% glucose and 0.5% ammonium sulfate. SD casa is SD supplemented with 0.2% casamino acids (Difco).

Plasmids

The P2047 *ADE4* mutant plasmid was constructed as follows : the mutant *ADE4* coding sequence was amplified from the *BRA11-2* [7] genomic DNA using oligonucleotides 48 and 429.

48 : 5' CGCGGATCCAAATGTGTGGTATTTTAG 3'

429 : 5' AAACTGCAGTCAATAATCTGCACAATTATATAAT C 3'

The resulting PCR fragment was digested with *Bam*HI and *Pst*I and introduced in the pCM189 vector [9] digested with *Bam*HI and *Pst*I. The resulting plasmid allows expression of the *ADE4* mutant gene under control of a tetracyclin regulatable promoter.

Preparation of cell extracts for enzyme assay

Harvested yeast was suspended in ca 3 vol of Tris/HCl buffer (pH 7.4) containing 1 mM EDTA and 10 mM 2-mercaptoethanol (Buffer A). The cells were disrupted by

vigorous shaking for 3 min with glass beads (ca 1 mm diameter) in a Pyrex test tube. The resulting solution was centrifuged at 100,000 g for 60 min. Supernatant was thoroughly dialyzed against ca 200 vol of buffer A overnight.

Enzyme assay

Phosphomonoesterases were assayed colorimetrically by measuring inorganic phosphate (Pi) released from various phosphomonoesters using the methods by Chen *et al.* [10]. Assays were performed at 37°C. The reaction mixture contained 100 mM imidazole/HCl buffer (pH 6.5), 25 mM $MgCl_2$, 0.1% BSA, 10 mM phosphomonoester substrate and appropriate amount of cell extracts. For IMP-specific 5'-nucleotidase assay IMP was used as a substrate. For assay of non-specific phosphatases, phenylphosphate or 1-glycerophosphate was used as a substrate. One unit of the enzyme activity corresponds to the release of 1 μmol of Pi/min from the substrate. Protein concentration was determined by the method of Lowry *et al.* [11] using crystalline BSA as a standard.

HPLC analysis of excreted purine compounds

Wild type and *isn1* strains transformed with P2047 were grown in adenine and uracil free SD casa medium. Cells were then harvested and the medium was filtered on 0.22 μm filter units. Separation of purine compounds of the medium was achieved by HPLC on a Supelcosil LC-18 5 μm reversed phase column using a step gradient set up with A buffer (0.01 M KH_2PO_4) and B buffer (20% A buffer – 80% methanol). The following proportions of A and B buffers, respectively (indicated in parentheses) were used at the indicated run time : 0 min (97/3), 13 min (89/11), 17 min (75/25), 19 min (30/70) and 27 min (97/3) and the flow rate was 1 ml/min. Excreted purine compounds separation was monitored by following absorbance at 260 nm at the column end and the different peaks obtained were identified by comparison with the retention time of known standards.

Authors' contributions

RI carried out Isn1p purification and enzymatic activities studies. CSM did the in vivo and HPLC studies. SC prepared the sample for mass spectrometry analysis. RK contributed to enzymatic activities studies. JMS did the mass spectrometry analysis and computer work to identify YOR155c. BDF designed the study, did the sequence alignements and organized the manuscript.

Acknowledgements

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References

1. Seegmiller JE, Rosenbloom FM and Kelley WN **An enzyme defect associated with a sex linked human neurological disorder and excessive purine synthesis** *Science* 1967, **155:**1682-1684
2. Giblet ER, Anderson AJ, Cohen F, Pollara B and Meuwissen HJ **Adenosine deaminase deficiency in two patients with severely impaired immunodeficiency** *Lancet* 1972, **2:**1067-1069
3. Guetsova ML, Lecoq K and Daignan-Fornier B **The isolation and characterization of *Saccharomyces cerevisiae* mutants that constitutively express purine biosynthetic genes** *Genetics* 1997, **147:**383-397
4. Lecoq K, Belloc I, Desgranges C and Daignan-Fornier B **Role of adenosine kinase in *Saccharomyces cerevisiae* : Identification of the *ADO1* gene and study of the mutant phenotypes** *Yeast* 2001, **18:**335-342
5. Lecoq K, Belloc I, Desgranges C, Konrad M and Daignan-Fornier B ***YLR209c* encodes *Saccharomyces cerevisiae* purine nucleoside phosphorylase** *J Bacteriol* 2001, **183:**4910-4913
6. Itoh R **Purification and some porperties of an IMP-specific 5'-nucleotidase from yeast** *Biochem J* 1994, **198:**593-598
7. Rebora K, Desmoucelles C, Borne F, Pinson B and Daignan-Fornier B **Yeast AMP pathway genes respond to adenine through regulated synthesis of a metabolic intermediate** *Mol Cell Biol* 2001, **21:**7901-7912
8. Laemmli UK **Cleavage of structural proteins during the assembly of the head of bacteriophage T4** *Nature* 1970, **227:**680-685
9. Gari E, Piedrafita L, Aldea M and Herrero E **A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*.** *Yeast* 1997, **13:**837-848
10. Chen PS Jr, Toribara TY and Warner H **Microdetermination of phosphorus** *Anal Chem* 1956, **28:**1756-1758
11. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ **Protein measurement with Folin phenol reagent** *J Biol Chem* 1951, **193:**21422-21430
12. Roepstorff P and Fohlman J **Proposal for a common nomenclature for sequence ions in mass spectra of peptides** *Biomed Mass Spectrom* 1984, **11:**601
13. Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M and Sakaki Y **A comprehensive two-hybrid analysis to explore the yeast protein interactome** *Proc Natl Acad Sci USA* 2001, **98:**4569-4574
14. Nakanishi T and Sekimizu K **SDT1/SSM1, a multicopy suppressor of S-II null mutant, encodes a novel pyrimidine 5'-nucleotidase** *J Biol Chem* 2002, **277:**22103-22106
15. Escobar-Henriques M and Daignan-Fornier B **Transcriptional regulation of the yeast GMP synthesis pathway by its end products** *J Biol Chem* 2001, **276:**1523-1530
16. Gallert KC, Ohanjan T, Daignan-Fornier B, Lottspeich F and Krauss G **Enzymatic properties and inhibition by single-stranded ARS sequences of adenylosuccinate synthetase from *Saccharomyces cerevisiae*** *Eur J Biochem* 1996, **239:**487-493
17. Jelinsky SA and Samson LD **Global response of *Saccharomyces cerevisiae* to an alkylating agent** *Proc Natl Acad Sci U S A* 1999, **96:**1486-1491
18. Ho Y, Gruhler A, Heilbut A, Bader GD, Moore L, Adams SL, Millar A, Taylor P, Bennett K and Boutilier K **Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry** *Nature* 2002, **415:**180-183
19. Prolla T., Christie DM and Liskay RM **Dual requirement in yeast DNA mismatch repair for MLH1 and PMS1, two homologs of the bacterial mutL gene** *Mol Cell Biol* 1994, **14:**407-415
20. Hill-Perkins M, Jones MD and Karran P **Site-specific mutagenesis in vivo by single methylated or deaminated purine bases** *Mutat Res* 1986, **162:**153-163