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RESEARCH REPORT

Heterologous Expression in Yeast of Human Ornithine Carriers *ORNT1* and *ORNT2* and of *ORNT1* Alleles Implicated in HHH Syndrome in Humans

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Abstract Hyperornithinemia–hyperammonemia–homocitrullinuria (HHH) syndrome is an autosomal recessive metabolic disorder usually presenting in the neonatal period with intermittent episodes of hyperammonemia, psychomotor delay, and progressive encephalopathy. Adult cases usually evolve into frank spastic paraparesis. The syndrome is caused by mutations in *SLC25A15*/*ORNT1* encoding the mitochondrial ornithine transporter; a second ornithine transporter, *ORNT2* of unknown function, is also present in most placental mammals. *ORNT2* is believed to originate from an ancient retro-transposition event. In yeast *Saccharomyces cerevisiae* the major function of the transporter (encoded by *Arg11*) is to shuttle ornithine from the mitochondrial matrix to the cytosol. Its inactivation abolishes growth in the absence of arginine.

In this work, we used functional complementation in *S. cerevisiae* to characterize the function of human *ORNT2* and to test the pathogenicity of *ORNT1* mutations found in HHH patients. Notably, we found that human *ORNT1* but not *ORNT2* complements the deletion of the yeast gene, despite their high level of homology. However, we identified some key residues in ORNT2, which may recover its functional competence when replaced with the corresponding residues of ORNT1, suggesting that roles of the two transporters are different. Moreover, we used this system to test a series of missense mutations of *ORNT1* identified in patients with HHH syndrome. All mutations had a detrimental effect on the functionality of the human gene, without however clear genotype–phenotype correlations. Our data support yeast as a simple and effective model to validate missense mutations occurring in patients with HHH.

Abbreviations

HHH	Hyperornithinemia–hyperammonemia–homocitrullinuria
ORNT1	Ornithine transporter 1
ORNT2	Ornithine transporter 2
ORNT3	Ornithine transporter 3
OTC	Ornithine transcarbamylase

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Introduction

Hyperornithinemia–hyperammonemia–homocitrullinuria (HHH; MIM #238970) syndrome is a rare autosomal recessive disorder of urea and ornithine metabolism. It usually manifests in the first years of life, although few patients with later-onset and a milder phenotype have been reported. The acute phase of the disease resembles that of other urea cycle defects with hyperammonemia, vomiting, ataxia, lethargy, and coma. The progression of the disease

includes psychomotor delay, blood clotting abnormalities, and intolerance to proteins. Patients display elevated plasma ornithine and ammonia that result in secondary homocitrullinuria (Valle and Simell 2001; Tessa et al. 2009). The disease is caused by mutations of the *SLC25A15* gene (MIM #603861, alternatively named *ORNT1*), encoding the mitochondrial ornithine transporter ORNT1 (Camacho et al. 1999), mainly expressed in the liver (Cheung et al. 1989; Fiermonte et al. 2003). Human ORNT1 is responsible for the electroneutral exchange of ornithine for citrulline or a hydrogen ion inside mitochondria (Fiermonte et al. 2003). In *Saccharomyces cerevisiae* the inactivation of the orthologue of *ORNT1* (*Arg11*) renders the cells auxotrophic for arginine (Crabeel et al. 1996; Soetens et al. 1998). To date, more than 30 different mutations have been reported to cause HHH syndrome (Camacho and Rioseco-Camacho 2012), but the correlation between the type of mutation and the patients' phenotype is still unclear (Tessa et al. 2009). Most placental mammals possess also a second gene, *SCL25A2* or *ORNT2* (MIM #608157), an intronless gene probably derived from a retro-transposition event of *ORNT1*. The high level of identity (87%) between the ORNT1 and 2 proteins suggested that ORNT2 could act as a second mitochondrial ornithine transporter, possibly explaining the milder phenotype observed in HHH patients, compared to other urea cycle disorders (Camacho et al. 2003). Nevertheless, the cellular role(s) of the human ORNT2 protein is not precisely established yet. Another gene of the same family, *SLC25A29*, encodes a mitochondrial transporter that was also proposed to vicariate the function of ORNT1 and was named ORNT3 (Camacho and Rioseco-Camacho 2009). However, this protein presents less than 50% of sequence similarity and a recent work showed that *SLC25A29* transports preferentially lysine and arginine and to a much lesser extent histidine and ornithine when expressed in phospholipid vesicles (Porcelli et al. 2014).

In this work we employed *S. cerevisiae* to study the function of missense mutations in *ORNT1* gene and to better dissect the role of *ORNT2*.

Materials and Methods

Yeast Strain and Media

Δ*Arg11 Y02386* (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *YOR130c*::*KanMX4*) was purchased from Euroscarf Consortium. Yeast strain was cultured as previously described (Trevisson et al. 2009). All yeast DNA transformations were performed with the PEG–lithium acetate method (Chen et al. 1992).

Construction of Yeast Expression Vectors

The entire coding region of *Arg11*, *ORNT1*, and *ORNT2* were amplified using specific oligonucleotides, cloned into the yeast multi-copy expression vector pYES2.1/V5-His-TOPO (Invitrogen) and mutagenized as previously described (Trevisson et al. 2009).

Proteins Extraction and Immunoblot

Proteins were precipitated and separated as previously described (Trevisson et al. 2009). Human ORNT1 was detected with 1:1,000 rabbit anti-human ORNT1 (GeneTex) and a 1:10,000 HRP-conjugated goat anti-rabbit antibody (Santa Cruz) by enhanced chemiluminescence (Amersham Pharmacia Biosciences). As loading control, the membrane was probed with mouse anti-yeast Porin antibody (Mito-Sciences).

Molecular Modeling

Images were generated using the PyMOL software and as templates for modeling AAC1 (Protein Data Bank [pdb] code 1OKC) as previously reported (Tessa et al. 2009). Sequence alignment was performed using *Multialin* software (http://multalin.toulouse.inra.fr/multalin/).

Results

Human ORNT2 Does Not Rescue the Phenotype of ΔArg11 Strain

To better dissect the role of human ornithine transporters, we expressed them in *S. cerevisiae*. The deletion of the mitochondrial ornithine transporter *Arg11* in *S. cerevisiae* results in the loss of ability to growth in absence of arginine (Crabeel et al. 1996). *ORNT1* can complement the deletion of *Arg11* (Ersoy Tunali et al. 2014). Considering the high level of homology between ORNT1 and ORNT2 and the recently published biochemical data on SLC25A29 (Porcelli et al. 2014), we decided to test only ORNT2. As shown in Fig. 1a, the overexpression of human *ORNT1*, but not *ORNT2*, can rescue the growth phenotype of the deleted strain in selective medium lacking arginine.

Arginine 179 Is Critical for the Different Function of ORNT1 and ORNT2 in Yeast

We were surprised by the inability of *ORNT2* to complement the deleted yeast strain, since the analysis of the primary structure revealed that the two human proteins

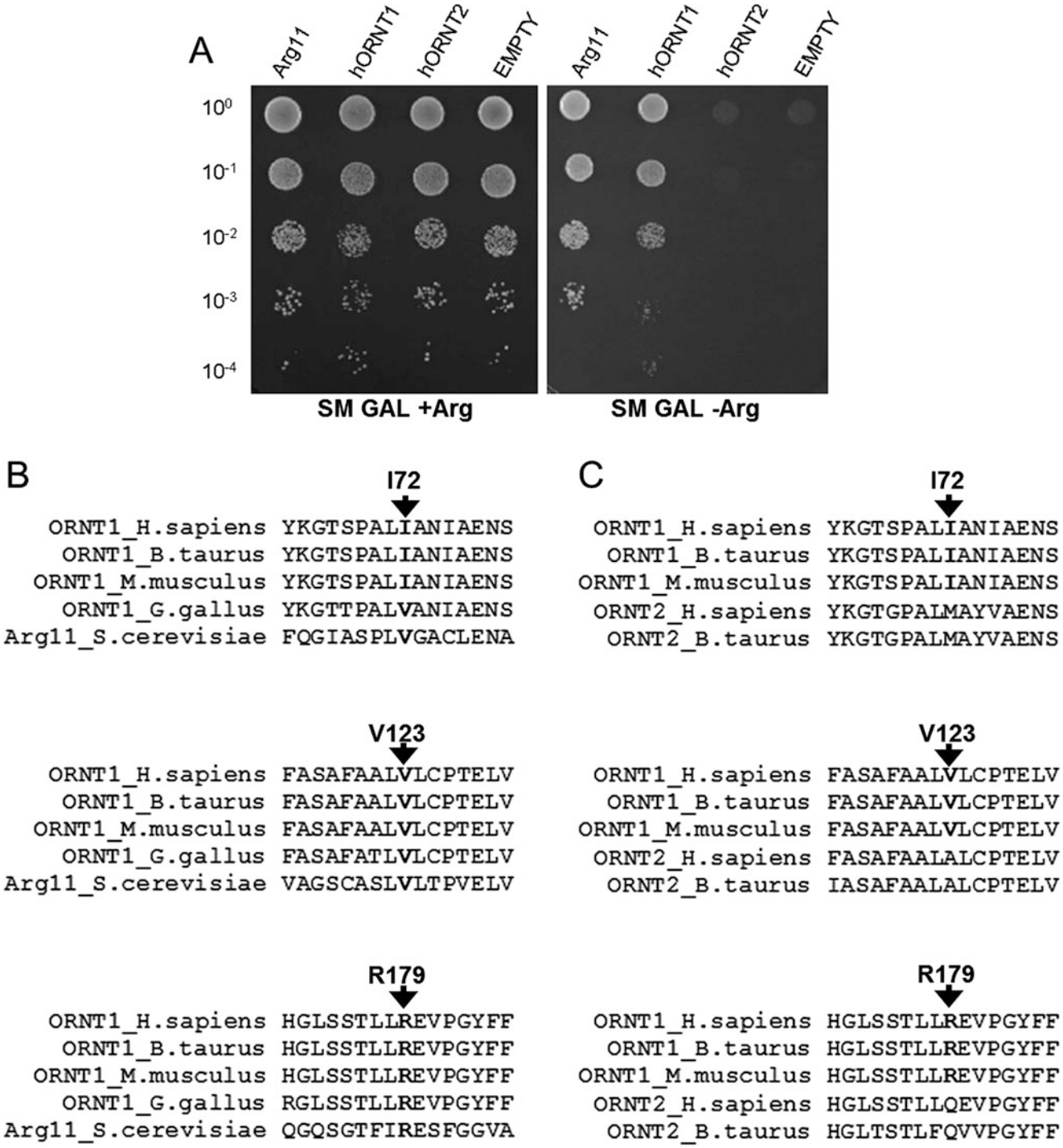

Fig. 1 (**a**) Functional complementation of Δ*Arg11* strain transformed with the high-copy pYES2.1 vector expressing wild-type yeast *Arg11* human *ORNT1* or *ORNT2* on minimal selective medium containing galactose as carbon source and depleted of arginine (SM GAL − Arg). Cells were grown 2 days on liquid medium at 30°; then an initial suspension at 1 U OD600 nm/mL was diluted 1/10 four times and spotted. Plates were incubated at 30° for 4 days. Growth in the presence of arginine (SM GAL + Arg) was performed as positive control. EMPTY: ΔArg11 transformed with empty pYES2.1 vector. (**b**) Alignment between ORNT1 and ORNT2 amino acidic sequences (*top*) and between ORNT1 from different species (*bottom*). *Black arrows* indicate the amino acids that are not conserved; *red arrows* indicate those that are conserved among species but not between ORNT1 and ORNT2. Alignment was performed using *Multalin* software (http://multalin.toulouse.inra.fr/multalin)

have a very high degree of identity (87%) and similarity (92%). Therefore, among the 38 amino acids that are discordant between the two proteins, we focused on three particular residues (isoleucine 72, valine 123, and arginine 179) that are highly conserved among ORNT1 orthologues, but have diverged in ORNT2 (methionine 72, alanine 123, and glutamine 179) (Fig. 1b). We thus replaced the three specific residues of ORNT2 with the corresponding amino acids present in ORNT1, introducing either single or multiple substitutions, and checked the growth in non-permissive conditions of yeast cells expressing the mutant isoforms of ORNT2. Our functional assay (Fig. 2a) shows that, among the single substitutions, only the p.Gln179Arg partially rescues the phenotype of Δ*Arg11* strain, while no growth could be detected for both single and double substitutions p.Met72Ile and p.Ala123Val. However, when p.Gln179Arg is coupled with either p.Met72Ile or p.Ala123Val, the growth phenotype markedly ameliorates, and cells expressing the triple mutant ORNT2 (p.Gln179Arg/p.Met72Ile/p.Ala123Val) display a growth rate similar to those expressing the wild-type ORNT1 (Fig. 2a).

The reciprocal substitutions on the human *ORNT1* gene confirmed the crucial role of residue 179 as the change of arginine into glutamine significantly reduces the ability of *ORNT1* to rescue the Δ*Arg11* phenotype while the other two amino acidic changes exacerbate the phenotype of the p.Arg179Gln substitution when expressed together, with specular results to those previously obtained for the ORNT2 assay (Fig. 2b). Western blot analysis of ORNT1 steady-state protein levels showed that wild-type and mutant p.Arg179Gln are expressed at comparable levels, whereas only the triple mutation p.Arg179Gln/p.Val123Ala/p.Ile72Met actually affects the stability of ORNT1 protein (Fig. 2c).

HHH Mutations Abolish the Ability of hORNT1 to Rescue Arg11 Deletion

Because human *ORNT1* could rescue the phenotype of Δ*Arg11* and a recent publication showed that a single HHH mutation had a detrimental effect on the gene function when expressed in yeast (Ersoy Tunali et al. 2014), we wondered whether this system could be employed to validate a set of missense mutations found in patients with HHH syndrome. We therefore expressed in the yeast system a series of mutations already identified in HHH patients (Camacho et al. 1999; Fecarotta et al. 2006; Tessa et al. 2009), as well as an additional variant (p.Gln246Lys) found *in cis* in a single adult patient (case #16 in Tessa et al. 2009 (Tessa et al. 2009)) and thought to act as a modifier (unpublished). We found that all the mutants analyzed, with the only exception of p.Gln246Lys, partially or completely abolish the ability of *ORNT1* to rescue the phenotype of the deleted strain. Only the p.Met37Arg and p.Leu71Gln alleles retained partial residual ability to grow in non-permissive conditions (Fig. 3a).

Analysis of the steady-state protein levels revealed that all mutations tested are relatively unaffected in their stability, with the exception of p.Ile272Phe and p.Gly113Cys that were present in lower amounts, suggesting a detrimental effect on the protein structure possibly promoting its faster degradation (Fig. 3b).

The p.Gln246Lys allele had no significant effect on yeast growth and protein levels suggesting that per se it has a neutral consequence on ORNT1 function. Since this variation was found *in cis* with the p.Ala70Leu mutation, we constructed a double mutant p.Ala70Leu; p.Gln246Lys. We found that this mutant behaves like the p.Ala70Leu allele, supporting the neutral nature of this variant (Fig. 3c).

Discussion

The HHH syndrome is characterized by multiple defects in different cellular processes. The metabolism of urea and ornithine is primary impaired, since the disease is caused by mutations of the *SLC25A15* gene, encoding the mitochondrial ornithine transporter ORNT1. More than 30 missense mutations linking *ORNT1* to HHH disease have been already reported and this number is growing.

In this work we have developed a yeast system to investigate the function of human ornithine transporters and to validate pathogenic mutations in *ORNT1* found in patients with HHH syndrome. Our results show that while human *ORNT1* complements Δ*Arg11* yeast (Ersoy Tunali et al. 2014), *ORNT2* does not. To explain the different functionality of the two proteins, we focused on three residues that are conserved in ORNT1 proteins among different species, but not in ORNT2. Among these, arginine in position 179 seems to be crucial for ORNT1 function. In fact its substitution with the glutamine present in this position in ORNT2 severely impairs its ability to rescue the Δ*Arg11* growth phenotype. The substitution of the other two residues (isoleucine 72 and valine 123) has little effect by themselves, but precipitate the effect of the p.Arg179Gln substitution. Conversely, when the ORNT1 residues were inserted in ORNT2, we observed a recovery of its ability to correct the Δ*Arg11* phenotype. Again the most critical substitution was the one affecting residue 179. Analysis of protein steady level revealed that the ORNT1 p.Arg179Gln substitution has no effect on stability, suggesting that it affects primarily the transport function. In fact, residue 179 is buried in the middle of the cavity (Fig. 2d, e) and its substitution with an arginine causes a change in the charge of the transmembrane domain (from 0 to −1). Similarly to what has been reported for the p.Glu180Lys mutation

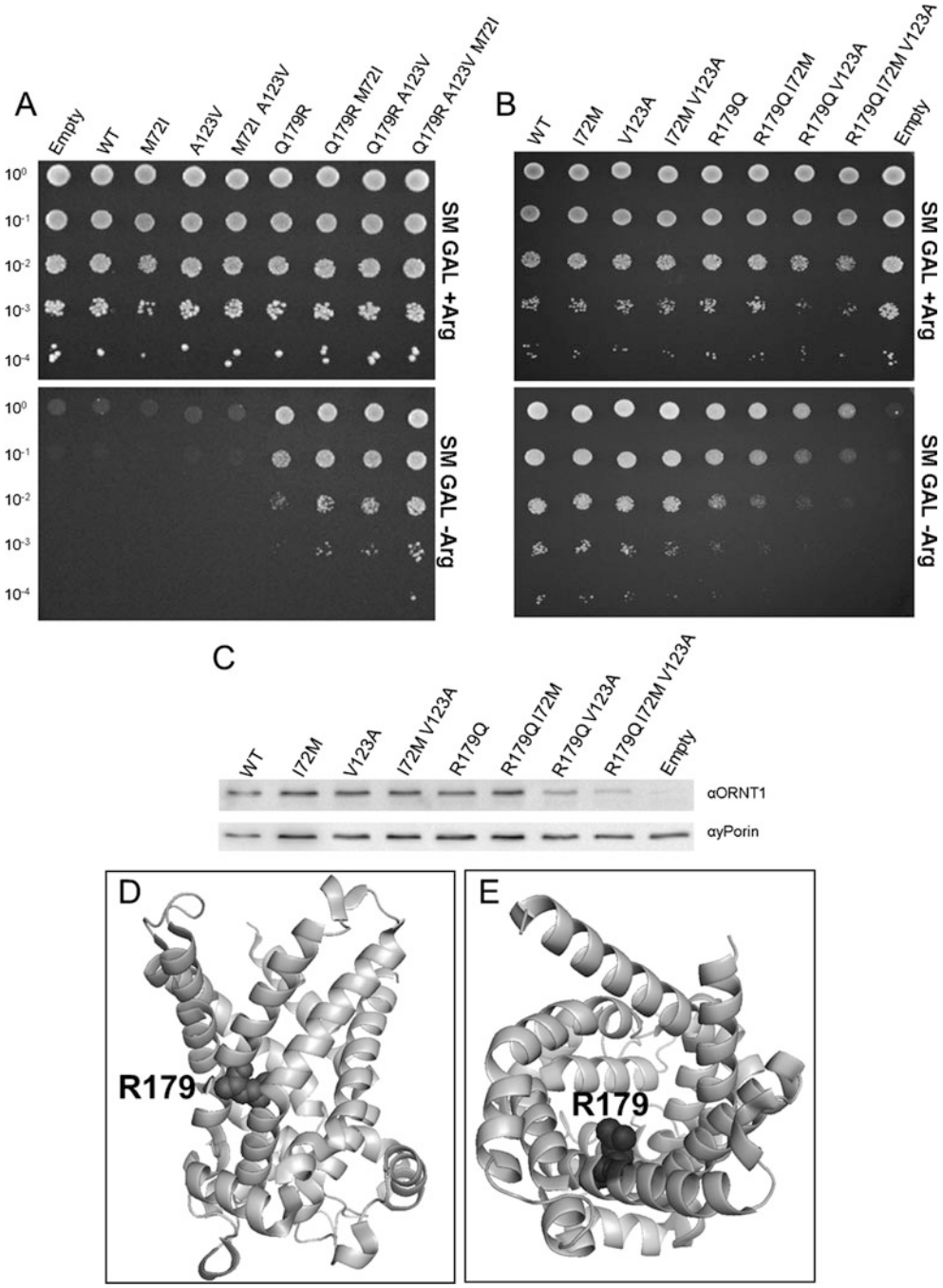

Fig. 2 (**a**) Functional complementation of the Δ*Arg11* strain transformed with the high-copy pYES2.1 vector expressing wild-type *ORNT2* or mutagenized to carry the residues of ORNT1. (**b**) Functional complementation of Δ*Arg11* strain transformed with the high-copy pYES2.1 vector expressing wild-type *ORNT1* or the gene mutagenized to carry the residues specific for ORNT2. The experiment was performed as described above. (**c**) Immunoblot analysis of equal amounts of total cell lysate extracted from Δ*Arg11* strain expressing human wild-type *ORNT1* or carrying the amino acidic changes described above. Immunoblot against yeast Porin (α-yPorin) was performed as loading control. Molecular modeling of residue p. Arg179 showed from the lateral side (**d**) or from the top (**e**), with the side chain exposed to the inner part of the pocket. AAC1 protein was used as template

(Wang and Chou 2012), p.Arg179Gln is likely to affect the substrate-binding pocket altering the affinity for ornithine. By contrast, the residues at positions 123 and 72 do not have per se an effect on protein function, although the presence concomitant with the p.Gln179Arg substitution reduces protein stability. Accordingly, a recent publication reveals that in reconstituted liposomes the residue 179 is crucial for substrates specificity in the two transporters

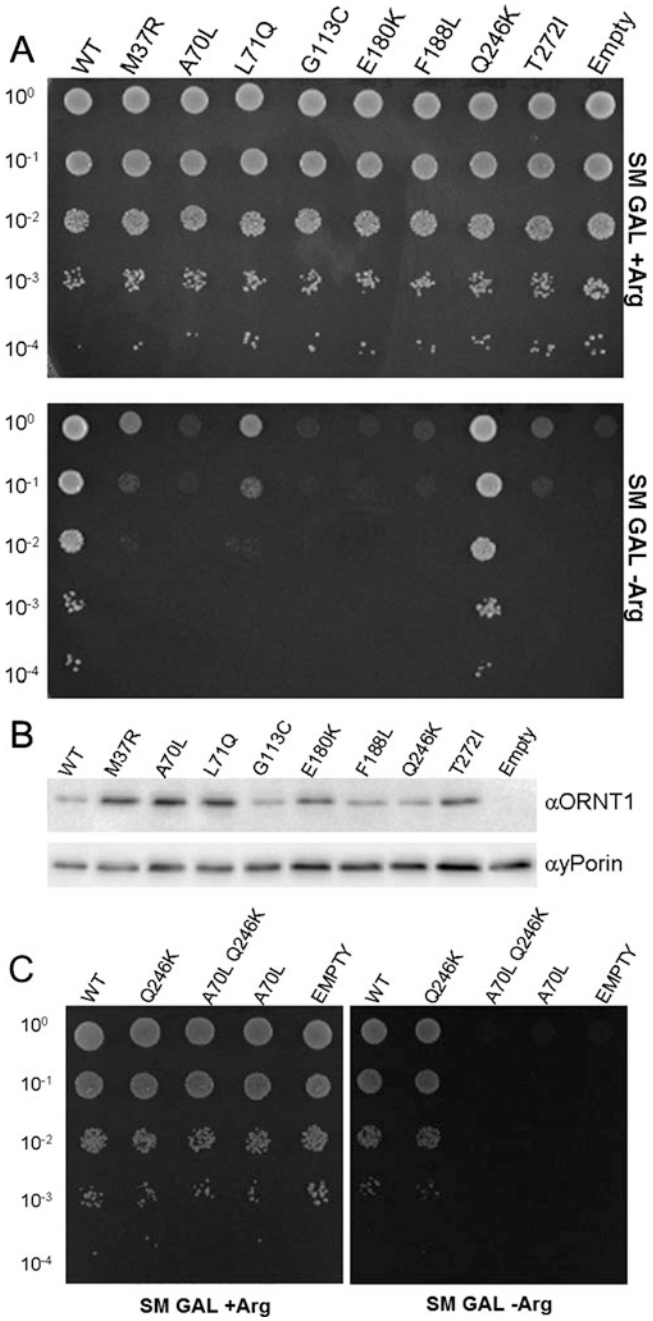

Fig. 3 Functional complementation (**a**) (**c**) and immunoblot (**b**) of Δ*Arg11* strain transformed with the high-copy pYES2.1 vector expressing wild-type ORNT1 or carrying mutations responsible for HHH syndrome. Experiments were performed as described above

(Monne et al. 2012). The presence of a glutamine in position 179 impairs the ability of ORNT2 to complement Δ*Arg11* yeast cells, suggesting that ORNT2 functions primarily as a transporter of compounds other than ornithine. Accordingly, biochemical characterization of recombinant ORNT2 indicated similar transport activities for ornithine, arginine, lysine, and histidine (without L/D selectivity), in contrast to ORNT1, endowed with higher substrate specificity. Furthermore, the enzymatic activity of recombinant ORNT2 (i.e., ornithine shuttling across reconstituted liposomes) was at least three times less than that of ORNT1 (Fiermonte et al. 2003). It is likely that ORNT2 could retain in vivo some residual ability to shuttle ornithine across the mitochondrial membrane, insufficient to rescue the viability of Δ*Arg11* yeast cells, but still able to improve ornithine metabolism in human fibroblasts carrying HHH mutations under overexpression conditions (Camacho et al. 2003). While this work was under review, another group shows the same result (Marobbio et al. 2015). The authors found that the lack of complementation

of the second transporter (SLC25A2 in the text) is due to a lower expression compared to the first one rather than to an inability to transport ornithine. However, our results clearly prove that the lack of complementation is due to the crucial role of residue 179 as the substitution of the arginine with the glutamine in the first transporter does not affect its expression levels.

In the second part of this work, we employed this model to validate the pathogenicity of HHH mutations found in patients and to examine possible genotype–phenotype correlations. This system was recently used to confirm the pathogenicity of a novel single HHH mutation (Ersoy Tunali et al. 2014); however we performed larger screening testing a number of gene variants located in different domains of ORNT1 protein and associated with different clinical phenotypes. The model was effective to functionally validate the role of disease-associated alleles. This strategy could be used for corroborating the effects of further gene variants associated in future HHH cases. It is interesting to notice that all the disease-causing alleles have a detrimental effect on the ability of the *ORNT1* to complement the deletion of *Arg11*, despite their different roles in cellular metabolism.

The different disease-causing mutations have been already analyzed using a computational three-dimensional model. The authors divide the mutations into three different classes, according to their location (Wang and Chou 2012). However the yeast assay gives more information than the computational analysis and mutations p.Ala70Leu and p. Leu71Gln that have been assigned to the same class by the computational data and are supposed to act with the same mechanism and in yeast behave differently, showing a different effect on the growth pattern (Fig. 3a).

Finally, the p.Ala70Leu mutation is due to the nucleotide c.208_209delGC/insTT and has been hypothesized that its pathogenic effect could be due either to an alteration of the splicing mechanism or to a functional effect on the protein but no experimental data was provided (Tessa et al. 2009). We now show that this mutation completely abolishes the ability of *ORNT1* to rescue the loss of function phenotype in yeast, suggesting that, independently from its possible effects on splicing, it has a detrimental effect on protein function.

Altogether these data suggested that yeast could be effectively used to analyze the pathogenicity of *ORNT1* mutations occurring in HHH patients and to discriminate neutral polymorphisms. Moreover, this system is less time-consuming than heterologous expression in bacteria and liposome reconstitution and, in contrast with the computational analysis of HHH mutants, it gives a functional in vivo results.

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Sentence Take-Home Message

Heterologous yeast expression of the human ornithine transporters reveals that ORNT2 cannot vicariate for the deletion of the yeast homolog suggesting that ORNT1 and ORNT2 have different functions determined by few key residues; moreover this system can be used to distinguish pathogenic ORTN1 mutations from neutral polymorphisms.

Compliance with Ethics and Guidelines

This article does not contain any studies with human or animal subjects performed by any of the authors.

Conflict of Interest Statement

Mara Doimo, Raffaele Lopreiato, Valentina Basso, Raissa Bortolotto, Alessandra Tessa, Filippo M. Santorelli, Eva Trevisson, and Leonardo Salviati declare that they have no conflict of interest.

Author Contribution

MD concepted, designed, and performed experiments, analyzed data, and drafted the article; RL concepted the experiments, analyzed data, and drafted the article; VB performed experiments; RB performed experiments; AT critically revised the article; FMS critically revised the article; ET drafted and critically revised the article; and LS concepted the experiment and drafted the article.

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