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DebaryOmics: an integrative –omics study to understand the halophilic behaviour of *Debaryomyces hansenii*

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Summary

***Debaryomyces hansenii* is a non-conventional yeast considered to be a well-suited option for a number of different industrial bioprocesses. It exhibits a set of beneficial traits (halotolerant, oleaginous, xerotolerant, inhibitory compounds resistant) which translates to a number of advantages for industrial fermentation setups when compared to traditional hosts. Although *D. hansenii* has been highly studied during the last three decades, especially in regards to its salt-tolerant character, the molecular mechanisms underlying this natural tolerance should be further investigated in order to broadly use this yeast in biotechnological processes. In this work, we performed a series of chemostat cultivations in controlled bioreactors where *D. hansenii* (CBS 767) was grown in the presence of either 1M NaCl or KCl and studied the transcriptomic and (phospho)proteomic profiles. Our results show that sodium and potassium trigger different responses at both expression and regulation of protein activity levels and also complemented previous reports pointing to specific cellular processes as key players in halotolerance, moreover providing novel information about the specific genes involved in each process. The phosphoproteomic analysis, the first of this kind ever reported in *D. hansenii*, also implicated a novel and yet uncharacterized cation transporter in the response to high sodium concentrations.**

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Introduction

Debaryomyces hansenii is a halophilic non-conventional yeast found in natural environments with high salt concentrations and/or osmotic pressure (e.g. seawater, soil, glaciers and salty food). It has been a model organism for the study of osmo- and salt tolerance mechanisms in eukaryotic cells over the last 30 years (Adler and Blomberg, 1985; Prista *et al*., 1997, 2005), and its genome was completely sequenced in 2004 (Dujon *et al*., 2004). *D. hansenii*'s halophilic behaviour has been fully established along the past 20 years, and it is proved that the presence of sodium in the medium protects the yeast cells against oxidative stress and additional abiotic stresses like extreme pH or high temperature (Almagro *et al*., 2000; Papouskova and Sychrova 2007; Navarrete *et al*., 2009; Garcia-Neto *et al*. 2017).

Several transport systems have been identified and hypothesized to have a significant role against osmotic stress and the maintenance of the cellular osmotic pressure in *D. hansenii* (as well as other yeast species), in the presence of salt. Examples of those are as follows: (i) H^+-ATPases, DhPma1 and DhVma2, located in the cell membrane and vacuole, respectively (Prista *et al*., 2005); (ii) DhNha1, an active Na^+/H^+-antiporter (Velkova and Sychrova 2006), and DhEna1, which actively secretes Na^+ by consuming ATP (Almagro *et al*., 2001); (iii) DhNhx1 and DhKha1, which are H^+/K^+-antiporters described to decrease the cytosolic Na^+ concentration by transferring Na^+ into vacuoles and the Golgi apparatus, respectively (Carcía-Salcedo *et al*., 2007; Montiel and Ramos 2007); and (iv) DhHak1, a K^+/H^+-symporter that in the presence of Na^+ may function as a K^+/Na^+-symporter as well (Martínez and Sychrova, 2011), and the K^+-transporter DhTrk1 (Prista *et al*., 2007).

In a recent study (Navarrete *et al*., 2020), our research group performed a physiological characterization of *D.*

hansenii (strain CBS 767) under high-salinity conditions in controlled bioreactors and confirmed that the most beneficial effects of salt (especially sodium) appear after long cultivation times, at least 20–24 h in batch cultures, almost 200 h under glucose limitation (0.2%) and between 100 and 240 h on solid media This study was a novel approach compared with previous works, where the salt effect was studied only 2–3 h after cultivation, and therefore lead to mistakenly ascribe a detrimental effect of salt on *D. hansenii*'s performance.

In the last two decades, several works studying the changes in *D. hansenii*'s metabolism due to salt exposure have been published. A limited number of proteomics/transcriptomics studies are also available, but with different strains used, experimental conditions and sampling regimes, making these studies neither comparable among each other nor accurate in obtaining conclusive results.

Gori *et al*. performed one of the first proteomic approaches in *D. hansenii* (strain CBS 767) in 2006. In that work, the authors investigated the changes in the yeast proteome upon exposure to NaCl (8% and 12% w/v), identifying a number of induced proteins involved in the synthesis of glycerol and the upper part of the glycolysis. On the other hand, proteins related to the lower part of the glycolysis, Krebs cycle and synthesis of amino acids were repressed. This study was carried out in YNB medium (pH 5.3) with 10 g l^{-1} of glucose as a carbon source, and the yeast cells were grown in shake flasks at 25°C. The samples for proteomic analysis were taken after 3 h from the addition of salt.

D. hansenii's proteome was also studied in the presence of KCl and after potassium starvation (Martínez and Luna, 2012). In this work, the authors identified upregulated proteins that were involved in stress responses, protein degradation and sterols synthesis after addition of 50 mM of KCl. Instead, proteins related to the upper part of the glycolysis, Krebs cycle and synthesis of amino acids were inhibited. Although the authors used the same *D. hansenii* strain (CBS 767) and medium (YNB) as Gori *et al*., in this case the pH was adjusted to 5.8, glucose was added as 20 g l^{-1} and the cells were cultivated in flasks at 28°C. Also differently, the samples for the proteomic analysis were taken after 2 h of KCl exposure.

Other research groups have studied the changes in the metabolism of *D. hansenii* under osmotic pressure. For example, the co-action of osmotic pressure and high temperature in *D. hansenii*'s growth (strains CBS 767 and CBS 1793) were studied in flasks and on plates by Papouskova and Sychrova in 2007. In this work, the authors demonstrated that the yeast growth stimulation by NaCl was temperature-dependent, testing a broad range of temperatures (from 23 to 37°C). The CBS 767 strain showed better growth at temperatures lower than 30°C in control conditions, while lower growth rates were observed at 23°C compared with 30°C when 0.6 M of NaCl was added. The CBS 1793 strain behaved similarly although it showed a higher tolerance in general. In this study, the cells were cultivated in liquid medium for a longer period, up to 50 h, so a beneficial effect of NaCl on *D. hansenii*'s growth was indeed observed, in agreement with Navarrete *et al*. (2020). Regarding the rest of growth conditions used in this study, YNB and YPD containing 20 g l^{-1} of glucose were used. Finally, the co-action of salt/temperature was described as pH-independent when studied the effect on metabolism in YPD (pH 6.8) or YNB (4.6).

More recently, Ramos-Moreno and Ramos (2019) described the overlapping responses between salt exposure and oxidative stress in *D. hansenii* (strain CBS 767). The authors observed the induction of genes involved in oxidative stress, in fast response to 200 mM Na^+/K^+ 'shocks' (between 10 min and 1 h of exposure), and that both the exposure to salt and oxidative stress triggering agents produced an increase in the intracellular oxygen species (ROS) levels. It was also shown that transcription of genes related to osmotic changes was regulated by H_2O_2, affecting the accumulation of in-cell sodium. In this study, the yeast cells were cultivated in flasks at 26°C, in YPD medium with 20 g l^{-1} of glucose.

As evidenced by the extensive amount of different culture conditions and especially short salt exposure times, a more systematic approach to study metabolic changes, under the presence of salt, is needed. The most accurate way of study, the effect of an external aggression (in this case, exposure to high salt concentrations) on cell metabolism, is by using continuous cultivations (chemostats). In this particular bioreaction set-up, all process variables are stable, and cells are in steady state in which the growth rate and yields are constant. In this culture mode, all the changes in gene expression, protein abundance, metabolic fluxes, regulation, etc, are exclusively due to the changing triggering factor (presence of salt). Therefore, the results obtained are expected to be accurate and, more importantly, comparable among conditions. Continuous culture fermentations have important applications in industry, as they overcome many limitations observed in batch processes. This kind of cultivation starts as a batch culture, with the particularity that the exponential growth phase can be extended indefinitely through the continuous addition of fresh fermentation medium. The volume in the bioreactor is constantly maintained by incorporation of an overflow weir or similar device, and steady-state conditions prevail as long as the rate of microbial cell growth equals the rate at which the cells are moved out from the vessel (Waites *et al*., 2001).

In this work, we have performed a series of chemostat experiments where *D. hansenii* CBS 767 was grown in the presence of either NaCl or KCl (1 M). Cell samples taken at steady state were used to study the transcriptome, proteome and phosphoproteome of this halophilic yeast, leading to a better understanding of what occurs at a molecular level, and unveiling some of the specific genes/proteins involved in the cellular processes previously reported to play a key role in the response to high salt concentrations. A phosphoproteomic analysis has been performed for the first time for this yeast under these experimental conditions, revealing novel and valuable data in relation to the differential responses triggered by either sodium or potassium present in the environment.

Results

Changes in the transcriptome of D. hansenii *at high salt concentrations*

Figure 1A shows a principal component analysis of the different samples (control without salt and 1 M NaCl/1 M KCl). The three experimental conditions cluster separately, and the NaCl samples stand as the ones with the lowest variance between replicates, that is the most reproducible ones. When looking at the up- and down-regulated genes obtained from the differential gene expression analysis, from 6506 genes investigated, 198 and 209 were significantly up- and down-regulated respectively in the presence of NaCl, compared with the control, while 503 and 497 were up- and down-regulated respectively in the presence of KCl (Fig. 2A). Those numbers correspond to 6.25% and 15.37% of significant changes in gene expression for each condition, with respect to the total analysed gene pool.

If we exclusively look at the 15 most significant changes in gene expression (False Discovery Rate (FDR) < 0.05), we observe that in the presence of sodium, four genes code for proteins with unknown function or not yet characterized (Table 1). Moreover, the majority of the genes with increased expression are related to membrane transport processes (such as phosphate/H^+ symporter and phosphate transporter, zinc uptake and hexose transporter). On the other hand, genes coding for the ARO3 synthase (involved in tyrosine biosynthesis), PEBP protein (a modulator of intracellular signalling pathways) or a sporulation-specific chitinase are examples of genes with repressed expression when NaCl is present in the medium (Table 1).

In the presence of potassium, we observed three cases of gene products with unknown function or yet uncharacterized, among the 15 most significant changes in gene expression (Table 2). Once more, genes related to transport are especially present among the ones with an increased gene expression (high-affinity zinc transporter, hexose transporter and ferrioxamine B transporter, as examples). Apart from the previously mentioned examples (ARO3 synthase, PEBP and chitinase), genes coding for a component of the Rsp5p E3-ubiquitin ligase complex or a trans-aconitate methyltransferase also showed a decreased gene expression when KCl is present (Table 2).

If we only look at differentially expressed genes with a $-2>\text{logFC}>2$ (FDR<0.05), the list of significant ORFs is reduced to a total of 75 (NaCl) and 99 (KCl) (Tables S1 and S2, respectively). Moreover, we can observe that more than 88% (NaCl) and 91% (KCl) of the up-regulated genes correspond to a $2<\text{logFC}<5$ and only 11.76% (NaCl) and 8.47% (KCl) present logFC>5 (Fig. S1A). Instead, the 95.83% (NaCl) and 100% (KCl) of the down-regulated genes show a $-2>\text{logFC}>-5$ (Fig. S1B).

Interestingly, the transcriptomic analysis revealed the existence of a significant number of genes of unknown function. These ORFs were sometimes described as containing a specific domain or presenting weakly similarity to other organism´s genes (Tables S1 and S2). Taking into consideration just those ORFs with a $-2>\text{logFC}>2$ (FDR<0.05), the calculated percentage of genes with unknown function in *D. hansenii* was 48% (NaCl) and 55.56% (KCl) (Fig. S2).

Enrichment analysis of RNA-seq data

In order to identify global processes potentially involved in the response to high salt concentrations, we first performed a Gene Ontology (GO) enrichment analysis. The results identified 17 different GO:terms (Fig. 3) and how those are represented in each studied condition (e.g. 0.5 in the colour scale = 50% of genes in that group are significantly over-expressed, and -0.5 = 50% are significantly repressed).

In the presence of NaCl, GO:terms containing a moderate-to-high number of up-regulated genes were mainly: glycolytic processes (GO:0006096), cell wall (GO:0030446, GO:0009277) and oxidoreductase activity (GO:0016491) (Fig. 3). On the other hand, when KCl is present, we observed that the GO:terms containing a higher number of up-regulated genes were those related to riboflavin biosynthetic process (GO:0009231), ribosomes (GO:0005840,GO:0003735) and translation (GO:0006412) (Fig. 3). Also in the presence of KCl, pheromone-dependent signal transduction (GO:0000750) and protein targeting to the vacuole (GO:0006623) were the GO:terms with the highest amount of repressed genes.

A higher level of specification in the enrichment analysis was obtained when pathways from the Kyoto

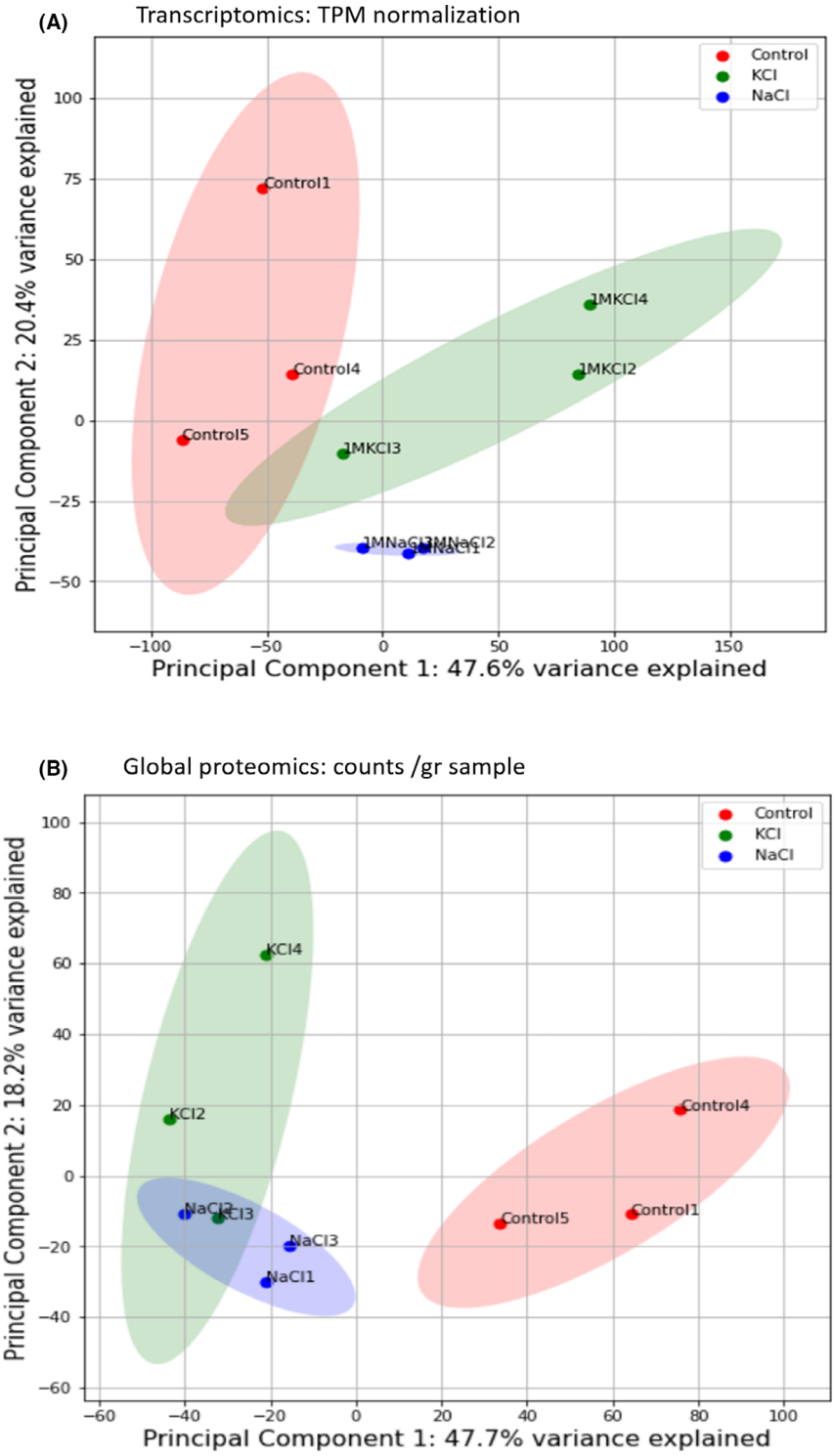

Fig. 1. Principal component analysis (PCA) of the replica samples used in the (A) transcriptomic study (TPM normalization) and (B) global proteomic study (normalized to counts/gr of sample).

Encyclopedia of Genes and Genomes (KEGG) were analysed (Fig. 4A). In this case, 21 pathways are changing in expression. In the presence of NaCl, pathways with a high number of up-regulated genes were glycolysis/gluconeogenesis (dha00010), steroid biosynthesis (dha00100), TCA cycle (dha00020), and pentose and

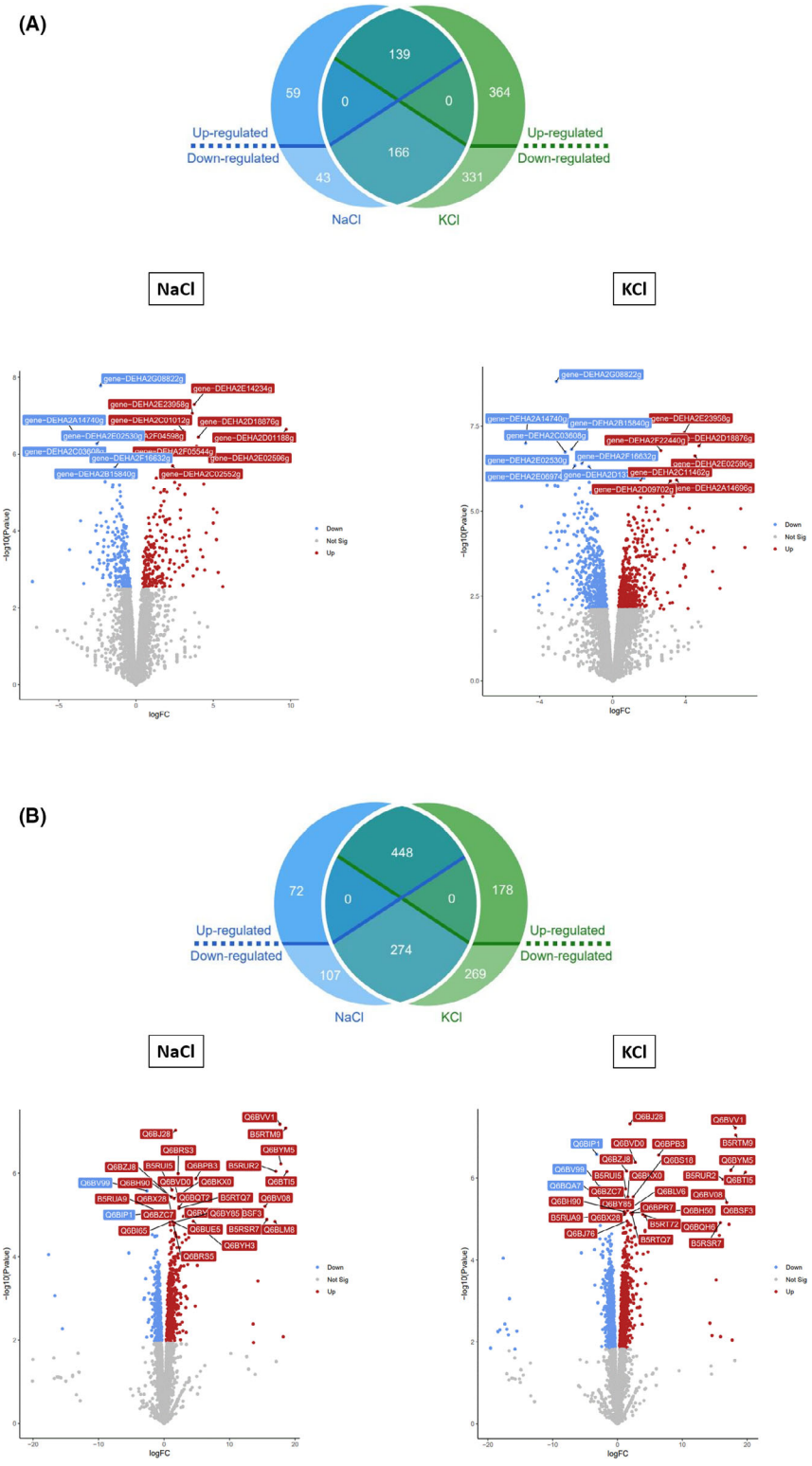

Fig. 2. Differential analysis obtained by using edgeR (FDR < 0.05).
A. Up: Venn diagram representing the number of differentially expressed genes in the presence of NaCl or KCl. Down: Volcano plots representing up- and down-regulated genes in NaCl or KCl respect to the control conditions. The 15 most significant genes are tagged in each figure.
B. Up: Venn diagram representing the number of differentially represented proteins in NaCl or KCl. Down: Volcano plots representing up- and down-represented proteins in NaCl or KCl respect to the control conditions. The 15 most significant proteins are tagged in each figure.

Table 1. The 15 most significant *D. hansenii*'s gene products in the presence of NaCl. The identified gene ID is coloured in red when up-regulated or in blue when down-regulated. Information about the corresponding gene product was obtained from GRYC (Genome Resources for Yeast Chromosomes) database at iGenolevures [GRYC - Home page (inra.fr)].

Gene ID	Gene product
DEHA2E14234g	PHO84 high-affinity inorganic phosphate/H+ symporter
DEHA2E23958g	ZRT1 High-affinity zinc transporter of the plasma membrane responsible for the majority of zinc uptake
DEHA2C01012g	Acid phosphatase precursor PHO2 gene
DEHA2D18876g	HXT5 Hexose transporter with moderate affinity for glucose
DEHA2F04598g	Unknown function
DEHA2D01188g	PHO89 Na^+- coupled phosphate transport protein
DEHA2F05544g	Unknown function
DEHA2E02596g	SIT1 Ferrioxamine B transporter member of the ARN family of transporters that specifically recognize siderophore-iron chelates
DEHA2C02552g	*Debaryomyces hansenii* ATPase ENA1p
DEHA2G08822g	ARO3 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase
DEHA2A14740g	Phosphatidyl Ethanolamine-Binding Protein (PEBP) domain
DEHA2E02530g	Uncharacterized protein involved in stress response, similar to tellurium resistance terD
DEHA2C03608g	BUL2 Component of the Rsp5p E3-ubiquitin ligase complex
DEHA2F16632g	CTS2 sporulation-specific chitinase
DEHA2B15840g	Unknown function

Table 2. The 15 most significant *D. hansenii*'s gene products in the presence of KCl. The identified gene ID is coloured in red when up-regulated or in blue when down-regulated. Information about the corresponding gene product was obtained from GRYC (Genome Resources for Yeast Chromosomes) database at iGenolevures (GRYC - Home page (inra.fr)).

Gene ID	Gene product
DEHA2E23958g	ZRT1 High-affinity zinc transporter of the plasma membrane responsible for the majority of zinc uptake
DEHA2D18876g	HXT5 Hexose transporter with moderate affinity for glucose
DEHA2F22440g	Unknown function
DEHA2E02596g	SIT1 Ferrioxamine B transporter member of the ARN family of transporters that specifically recognize siderophore-iron chelates
DEHA2C11462g	N6-adenine methyltransferase
DEHA2A14696g	SIT1 ferrioxamine B transporter
DEHA2D09702g	Cyclin, N-terminal domain
DEHA2G08822g	ARO3 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase
DEHA2A14740g	Phosphatidyl Ethanolamine-Binding Protein (PEBP) domain
DEHA2B15840g	Unknown function
DEHA2C03608g	BUL2 Component of the Rsp5p E3-ubiquitin ligase complex
DEHA2F16632g	CTS2 sporulation-specific chitinase
DEHA2E02530g	Uncharacterized proteins involved in stress response, similar to tellurium resistance terD
DEHA2D13750g	Aspartate aminotransferase (AAT) superfamily (fold type I) of pyridoxal phosphate (PLP)-dependent enzymes
DEHA2E06974g	TMT1 Trans-aconitate methyltransferase

glucuronate interconversions (dha00040). On the other hand, amino acid biosynthesis pathways (dha00220, dha00400 and dha01230) and biosynthesis of secondary metabolites (dha01110) are examples of pathways with high number of repressed genes (Fig. 4A).

Steroid biosynthesis (dha00100), ribosome (dha03010) and riboflavin metabolism (dha00740) were specially represented pathways among those containing high number of up-regulated genes in the presence of KCl. On the contrary, ascorbate and aldarate metabolism (dha00053), arginine biosynthesis (dha00220), valine, leucine and isoleucine degradation (dha00280) or histidine metabolism (dha00340) were among the pathways with the highest amount of repressed genes (Fig. 4A).

A deeper level of detail was achieved when KEGG modules, which are specific parts of a KEGG pathway, were analysed, although a much lower number of modules are normally identified as significant (five in our case). In this regard, glycolysis (dhaM00002 and dhaM00001) and gluconeogenesis (dhaM00003) are modules containing a very high number of up-regulated genes when sodium is present, whilst ergocalciferol biosynthesis (dhaM00102) and F-type ATPase (dhaM00158) modules are significantly up-regulated when potassium is present (Fig. 4B).

We also looked at every individual category described as significant (FDR < 0.05) and calculated the number of differentially expressed (DE) genes (up- or down-regulated) in relation to the total number of genes conforming the category (Figs S3-S5). We observed that 'transmembrane transport', 'oxidation–reduction process', 'catalytic activity' or 'translation' are among the most representative GO:terms in the presence of salt (Fig. S3). When the KEGG pathways were investigated, we found 'metabolic pathways' and 'biosynthesis of secondary metabolites' as the most representative ones (Fig. S4). Finally, KEGG modules like 'central carbohydrate metabolism', 'lipid metabolism' and 'energy metabolism' present the highest importance in the re-adjustment of the cells in the presence of salt (Fig. S5).

Global proteomic analysis of D. hansenii *at high salt concentrations*

A principal component analysis of the global proteomics samples (control without salt and 1 M NaCl/1 M KCl) was also performed to see their distribution and variance among the three conditions studied (Fig. 1B). The analysis revealed a clearly separate distribution of the control samples compared with when salt is present.

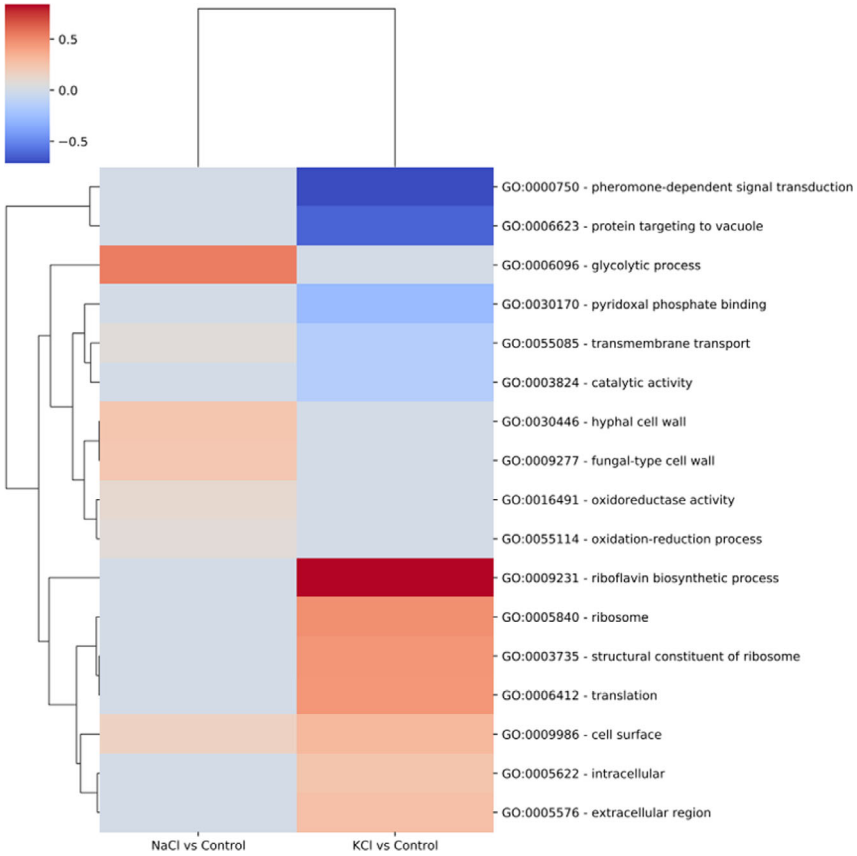

Fig. 3. Heatmap representing the most significant GO:terms in the presence of 1 M of salt (FDR < 0.05 and a minimum of five genes per group). The colour scale represents the percentage of genes in the corresponding group that are significantly up-regulated (in red) or down-regulated (in blue).

Additionally, the samples with NaCl were again the ones showing the lowest variance (highest reproducibility).

The differential analysis revealed a list of over- and down-represented proteins. From 3883 proteins investigated, 520 and 381 were over- and down-represented respectively in the presence of NaCl, compared with control, while 626 and 543 were over- and down-represented respectively in the presence of KCl (Fig. 2B). These numbers represent to 23.2% and 30.1% of significant changes in that specific proteins´ abundance for each condition, with respect to the total number of analysed proteins.

If we exclusively look at the 15 most significant changes identified in our protein list (FDR<0.05), we find the HXT5 hexose transporter (B5RTM9), a haloperoxidase (Q6BYM5) and a ferric oxidoreductase (Q6BV08) among the top ones (Table S3). Moreover, 6 of those 15 proteins show a logFC > 10. In the presence of KCl, apart from the aforementioned proteins which also show up in the 15 most significant protein changes, we find a NADPH dehydrogenase (Q6BTI5), several oxidoreductases (Q6BS18, Q6BV08) and a ubiquitin-conjugating enzyme (B5RUR2) (Table S4). We found 6 of those 15 proteins with a logFC > 10.

If we again exclusively search for proteins with a −2>logFC>2 (FDR < 0.05), we obtain a total of 82 proteins (NaCl) and 104 proteins (KCl) (Tables S3 and S4, respectively). Moreover, we can observe that more than 77% (for both NaCl and KCl) of the up-represented proteins show a 2<logFC < 5, only 1.4% (for both NaCl and KCl) show a 5<logFC<10, and 20.59% (NaCl) or 21.43% (KCl) presents logFC > 10 (Fig. S6A). Instead, the 71.43% (NaCl) and 67.65% (KCl) of the down-represented proteins show a −2>logFC>−5, only 7.14% (NaCl) and 2.94% (KCl) show a −5>logFC>−10, and 21.43% (NaCl) or 29.41% (KCl) presents logFC < 10 (Fig. S6B).

The existence of a vast number of uncharacterized proteins was also observed in the proteomic analysis. Sometimes, several of these proteins were also described as containing a specific protein domain or presenting weakly similarity to other proteins/enzymes described for other organisms (Tables S3 and S4). Taking into consideration just the ones presenting a

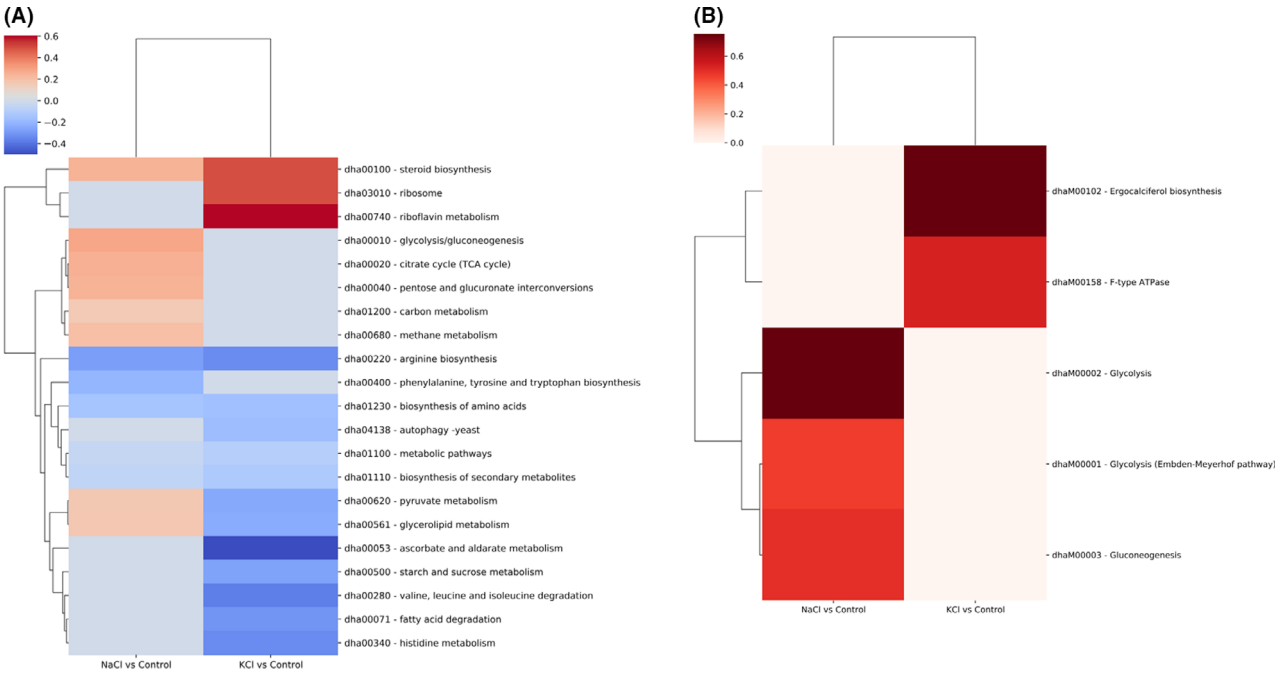

Fig. 4. Heatmaps representing the most significant KEGG pathways (A) and KEGG modules (B) in the presence of 1 M of salt (FDR < 0.05 and a minimum of five genes per group). The colour scale represents the percentage of genes in the corresponding group that are significantly up-regulated (in red) or down-regulated (in blue).

−2>logFC>2 (FDR < 0.05) in the differential analysis, the calculated percentage of proteins with unknown function in *D. hansenii* was 10.98% (NaCl) and 16.35% (KCl) (Fig. S2).

Enrichment analysis from the global proteomic data

The enrichment analysis obtained from the global proteomic data showed only six significant KEGG pathways in the presence of NaCl (FDR < 0.05), whilst five significant GO:terms and 1 significant KEGG pathway in the presence of KCl (Table 3). In both cases, down-regulated GO:terms or KEGG pathways were more abundant than up-regulated ones.

Table 3. Significant GO:terms and KEGG pathways obtained from the enrichment analysis of the global proteomic data from *D. hansenii* in the presence of 1 M salt (FDR<0.05). The identified term/pathway is coloured in red when up-regulated or in blue when down-regulated.

Condition	GO:term / KEGGpathway
1 M NaCl	Ribosome (dha03010)
	Biosynthesis of amino acids (dha01230)
	2-oxocarboxylic acid metabolism (dha01210)
	Base excision repair (dha03410)
	Lysine biosynthesis (dha00300)
	Arginine biosynthesis (dha00220)
1 M KCl	Oxidoreductase activity (GO:0016491)
	Nucleoside metabolic process (GO:0009116)
	Intracellular protein transport (GO:0006886)
	Ran GTPase binding (GO:0008536)
	Membrane (GO:0016020)
	Biosynthesis of unsaturated fatty acids (dha01040)

Identification of differentially phosphorylated proteins upon high salinity

A follow-up phosphoproteomic analysis was performed on the protein samples, in order to find which of the metabolic responses observed in the presence of salt was not just consequence of induction/repression of gene expression, but caused by specific regulation of the activity of key-specific enzymes. In addition, this analysis provided evidence of which specific enzymes can be considered as main targets of such regulation, as to date no information is available about this.

A general higher phosphorylation trend is observed when NaCl is present, compared with KCl (Fig. 5). Interestingly, only proteins Q6BHH1, Q6BI94, Q6BKG7 and Q6BX19 increased their abundance in the global analysis and, at the same time, showed a higher phosphorylation status in the presence of NaCl. Additionally, Q6BUR3 exhibited an extremely high phosphorylation status of the peptide '_NEHDES[Phospho (STY)]DFEIPDIDVGDDS[Phospho (STY)]DDE_', which is responsible for 'Pre-RNA processing' and residing at the end of the protein (Fig. 5A). Very interestingly, our analysis showed that a novel (yet uncharacterized) membrane cation transporter (B5RUG0) is involved in the response to high

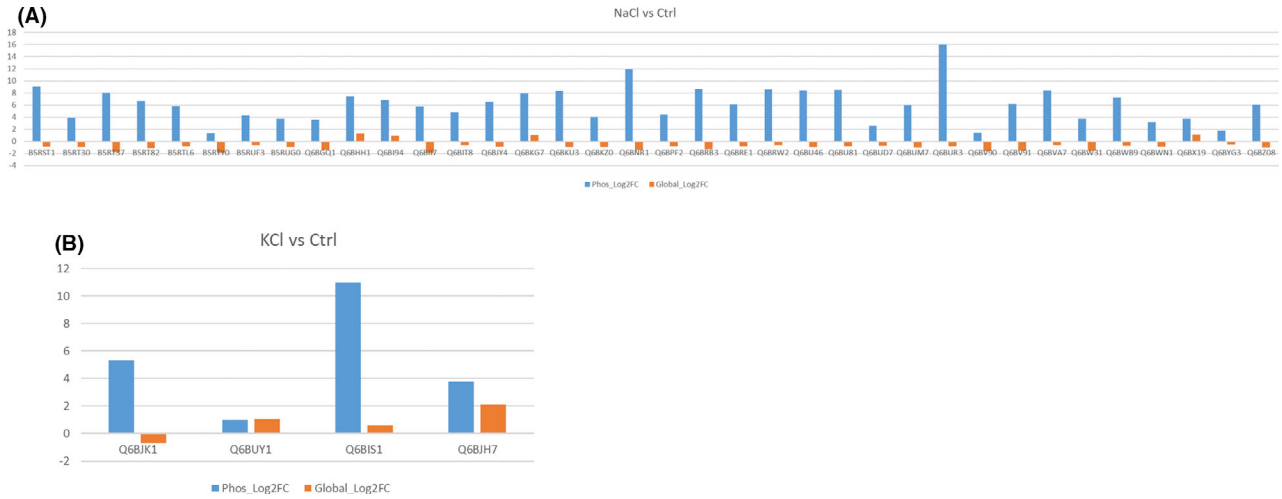

Fig. 5. Correlation between abundance patterns of phosphopeptides/proteins in the presence of salt. Log2FC values for the significant phosphopeptides/proteins obtained in the analysis are represented in the figure in (A) NaCl or (B) KCl.

sodium. None of the previous studies on *D. hansenii*'s had ever before reported the existence of this particular transporter, much less its involvement in the response to sodium.

In contrast, only four proteins were identified to have significant changes in their phosphorylation pattern when KCl is present. Moreover, the analysis showed a substantial increase of Q6BIS1 phosphorylated peptide '_FLS[Phospho (STY)]EEEAK_', a thioredoxin reductase at the C-term of the protein (Fig. 5B).

A complete list of the significant proteins obtained from the phospho-analysis can be found in Table 4 (NaCl) and Table 5 (KCl).

It is worth mentioning that out of 36 sodium responsive proteins found in the phosphoproteomic analysis, 13 are either unknown or uncharacterized proteins (36% of the total), which is a considerably high amount. In the case of the response to potassium, two out of the total four responsive proteins are in this category (50%).

Membrane transporters and osmo-regulation in D. hansenii

In order to investigate whether or not the genes coding for the most studied transport systems involved in osmoregulation in *D. hansenii* suffered any important changes in expression, we specifically searched for them during the transcriptomic analysis. In the presence of sodium, *DhENA1* (DEHA2C02552g) and *DhKHA1* (DEHA2F27280g) genes were up-regulated (FDR<0.05 and logFC of +2.36 and +1.98, respectively) (Fig. 6). Likewise, when potassium was present in the medium, these two genes were also up-regulated (FDR < 0.05 and logFC of +1.62 and +1.77 respectively). On the other hand, changes in gene expression were also observed for *DhNHA1* (DEHA2F05984g), *DhNHX1* (DEHA2G05280g) and *DhPMA1* (DEHA2A08800g) genes (FDR<0.05 and logFC of +0.42, −0.55 and +0.82, respectively) in the presence of KCl (Fig. 6). Interestingly, and in relation to the activity of Pma1, although we did not observe significant changes in its expression, the activity of this pump did probably increase in the presence of both NaCl or KCl, since the expression of *GPR1* (a negative regulator of Pma1 activity) was repressed about −16-fold compared with control in the presence of either salt.

Unfortunately, *DhHAK1* gene (DEHA2E08395g) was not among the annotated genes for *D. hansenii* in the available database nor in the ontologies file. The other transport systems investigated, *DhTRK1* (DEHA2A10340g) and *DhVMA2* (DEHA2B01892g), did not change their abundance in the conditions studied in this work (Fig. 6). The particular case of *DhHAL2* gene (DEHA2E21274g), coding for a nucleotidase involved in DhEna1 regulation, was also studied. Yet again, no changes in this protein were observed in any of our conditions (NaCl or KCl).

A comparison with salt responsive genes previously identified in Saccharomyces cerevisiae

During the last two decades, gene expression changes in *S. cerevisiae* have been investigated as part of the yeast response and adaptation to saline stress. Importantly to be reminded, *S. cerevisiae* is not considered a halotolerant yeast, and therefore, it suffers high stress already in the presence of low-medium salt concentrations. Different microarray analyses in *S. cerevisiae* have

Table 4. Significant proteins (and their phosphopeptides) identified in the phosphoproteomics analysis, in the presence of NaCl. The ID of each protein is also included in the table. Information about the proteins was obtained from UniProt database at (UniProt).

Protein ID	Peptide	Protein description
Q6BUR3	_NEHDES[Phospho (STY)]DFEIPDIDVGDDS[Phospho (STY)]DDE_	RIX1. rRNA processing
Q6BNR1	_NINNPS[Phospho (STY)]DLEEAIAR_	I-B22. Mitochondrial electron transport
B5RST1	_QASDDSISGLS[Phospho (STY)]LDEK_	PlsC domain containing. Glycerolipid metabolism
Q6BRB3	_DSS[Phospho (STY)]PTAPTTSTSTSTSGATSVPSSNSWAAALSK_	DEF1. RNA polymerase II degradation factor 1
Q6BRW2	_EQS[Phospho (STY)]PDSTILVSK_	Uncharacterized protein
Q6BU81	_ADEEEEDS[Phospho (STY)]DFVADENNDENDK_	Ubiquitinyl hydrolase 1
Q6BVA7	_AELES[Phospho (STY)]LFK_	SAR1. Transport vesicles from the ER
Q6BU46	_TGADSTDVSGS[Phospho (STY)]EQQPFQTPAFALFK_	DED81. Cytosolic asparaginyl-tRNA synthetase
Q6BKU3	_YQFENDS[Phospho (STY)]EDDEMEK_	SEC9. t-Snare protein
B5RT37	_IFNASSNS[Phospho (STY)]LSSM[Oxidation (M)]SGK_	Uncharacterized protein
Q6BKG7	_GPDDAEGFADAVDELQS[Phospho (STY)]NQLDTK_	Uncharacterized protein
Q6BHH1	_SSIDDHEEDS[Phospho (STY)]TEETEEPALIK_	ABP1. Actin binding protein
Q6BWB9	_IDDDDVYS[Phospho (STY)]EIDK_	AIM21. Mitochondrial migration/acting filaments
Q6BI94	_EEPPADNDLEYES[Phospho (STY)]MDEAEC[Carbamidomethyl (C)]K_	Uncharacterized protein
B5RT82	_NIT[Phospho (STY)]PNASNDDLSVK_	Arf-GAP. ER to Golgi vesicle-mediated transport
Q6BJY4	_SQNPLINM[Oxidation (M)]ESSS[Phospho (STY)]K_	SMI1. Regulation of cell wall biosynthesis
Q6BV91	_YTSNNLVNDPEGS[Phospho (STY)]DDERGRER_	PRP45. Pre-mRNA-processing protein 45
Q6BRE1	_NSGGY[Phospho (STY)]DSENVEGSGNSLLR_	Uncharacterized protein
Q6BZ08	_EVSEVNNLAS[Phospho (STY)]ITS[Phospho (STY)]VPS[Phospho (STY)]VAVK_	Uncharacterized protein
Q6BUM7	_S[Phospho (STY)]SLFSRDNVVNNY_	Uncharacterized protein
B5RTL6	_FS[Phospho (STY)]IGNSLLGSYR_	Non-specific serine/threonine kinase
Q6BII7	_S[Phospho (STY)]DTPTPVPEAQIR_	Elongation of fatty acids related-protein
Q6BIT8	_VQDVDGPSNTSRENDLYAVAS[Phospho (STY)]NK_	Uncharacterized protein
Q6BPF2	_TM[Oxidation (M)]DNSAAQLPS[Phospho (STY)]PADSRAPS[Phospho (STY)]VEEK_	Non-specific serine/threonine kinase
B5RUF3	_LSSSAPDES[Phospho (STY)]EIS[Phospho (STY)]ALENVTNDSIK_	PWWP protein. Histone methylation
Q6BKZ0	_DLDLPESLQEDYESVINS[Phospho (STY)]ESESVR_	Uncharacterized protein
B5RT30	_DENNLS[Phospho (STY)]DNEVSSDY[Phospho (STY)]VHDLK_	Oxidoreductase. FAD-binding protein
Q6BW31	_TINS[Phospho (STY)]SNNLHLIK_	Transmembrane transporter activity
B5RUG0	_SPS[Phospho (STY)]TSALFNK_	Cation transmembrane transporter activity
Q6BX19	_QTIPS[Phospho (STY)]PPASTINTSPGAVFVPSASVPPPR_	Uncharacterized protein
Q6BGQ1	_T[Phospho (STY)]IVTPVIYQIR_	Nitric oxide dioxygenase. Flavohemoprotein
Q6BWN1	_SFLPTGTNTPTEEDELSTSSGAS[Phospho (STY)]EDDEIAS[Phospho (STY)]LPDK_	Fatty acid synthase subunit alpha
Q6BUD7	_QSTDDSAS[Phospho (STY)]IM[Oxidation (M)]DEQLNDIPREPQAK_	3-isopropylmalate dehydratase
Q6BYG3	_APYPVYESST[Phospho (STY)]PPPVFTQK_	Uncharacterized protein
Q6BV90	_DIDSISNSNEDEAES[Phospho (STY)]EEVQEEEDEENEDELRVGR_	WAC domain containing protein. Cell growth
B5RTY0	_EC[Carbamidomethyl (C)]IDVPALEEHDS[Phospho (STY)]DQSR_	Uncharacterized protein

Table 5. Significant proteins (and their phosphopeptides) identified in the phosphoproteomics analysis, in the presence of KCl. The ID of each protein is also included in the table. Information about the proteins was obtained from UniProt database at (UniProt).

Protein ID	Peptide	Protein description
Q6BIS1	_FLS[Phospho (STY)]EEEAK_	TRR1 thioredoxin reductase
Q6BJK1	_AT[Phospho (STY)]NVEEDSDEEDIEEDDDAFFHKK_	ATPase activity. Ribosome biogenesis
Q6BJH7	_NININS[Phospho (STY)]PPK_	RMM. RNA recognition motif
Q6BUY1	_DDAESEY[Phospho (STY)]ET[Phospho (STY)]DGEEIKK_	Uncharacterized protein

been described in several studies for this purpose, and as an example, genes involved in ion homeostasis and osmolytes biosynthesis have been found to be specifically activated (Dhar *et al.*, 2011; Yale and Bohnert 2001). Differential changes can be also observed in translation-related processes (e.g. coding for ribosomal proteins) and genes involved in regulation of the cell wall composition (Melamed and Pnueli, 2008; Dhar *et al.*, 2011). Moreover, a strong up-regulation is associated with genes involved in amino acid biosynthesis and nucleotide metabolism, as well as energy supply processes such as respiration. Lastly, cell defence and transport functions are also typically induced in the model yeast (Yale and Bohnert 2001). In this work, we have reviewed some of those significant

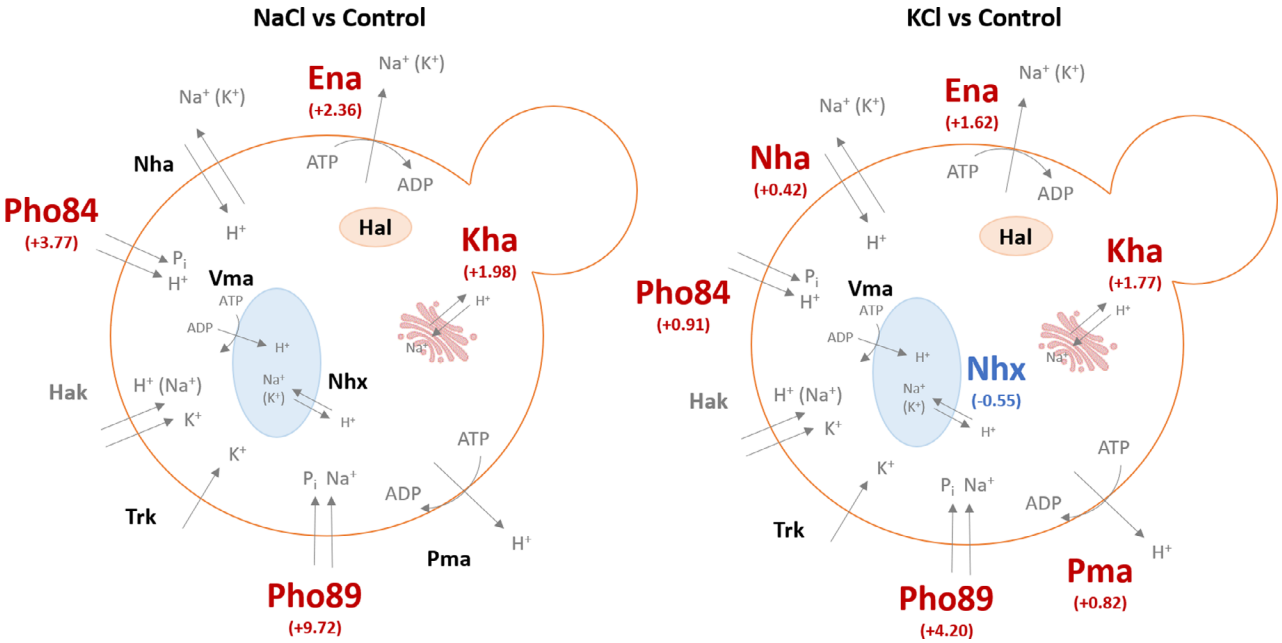

Fig. 6. Overview of the main transport systems involved in the maintenance of the cellular osmotic pressure in *D. hansenii*, and their changes at gene expression level in the presence of 1 M NaCl or KCl. Transport systems represented in red are significantly up-regulated while in blue, significantly down-regulated (FDR < 0.05 in both cases). The corresponding logFC value is specified in brackets under the name of the protein.

genes found to be important in *S. cerevisiae* saline stress response to see whether relevant expression changes are also observed in the response of *D. hansenii* to the presence of high salt (Table 6).

Thus, we searched for genes already well known for their physiological stress responses in *S. cerevisiae* as well as in other yeasts, such as *CTT1* (catalase T, with protected effect against hydrogen peroxide), *MSN4* (transcriptional factor, component of the stress-responsive system) and *HLR1* (involved in cell wall composition and integrity under osmotic stress) (Dhar *et al.*, 2011). Nevertheless, in our experimental conditions none of them experienced significant changes in their expression. In the particular case of *HLR1*, there was not even a homolog in *D. hansenii* (Table 6).

Genes involved in the synthesis and regulation of the cellular osmolytes glycerol (*GPD1/2*, *GPP1/2*) and trehalose (e.g. *GLK1*, *HXK1*, *TPS1/2*) have traditionally been targeted in RNA expression studies (Posas *et al.*, 2000; Yale and Bohnert 2001). In our case, we found up-regulation for *GPD1* in the presence of both NaCl and KCl, but only for *GPP1* in the presence of NaCl. Neither *GPD2* nor *GPP2* was identified in *D. hansenii*. In addition to *GPP1*, *D. hansenii* is endowed with an isoform of this glycerol-3-phosphatase, coded by *RHR2*, which is over-expressed both in the presence of sodium (+3.4-fold) and potassium (+4.3-fold) (Tables S1 and S2 respectively). None of the genes for synthesis/regulation of trehalose show differential expression changes in our conditions. Yet, again there was no homologous gene for *HXK1* found in *D. hansenii* (Table 6). However, a similar gene to *S. cerevisiae HXK2* was identified by BLASTn in *D. hansenii* (DEHA2F13992g), although it was not listed as significant in our study.

MET6/25 is genes associated with amino acid biosynthesis, found to be significantly up-regulated in *S. cerevisiae* under high salt stress (Yale and Bohnert 2001; Dhar *et al.*, 2011). However, none of them were found as significant in our differential analysis (Table 6).

Genes with roles in cell defence, ageing and cell death appear to be also up-regulated in *S. cerevisiae* in response to high salt (Yale and Bohnert 2001). One of those genes is *CUP1* (in its both versions *CUP1-1* and *CUP1-2*), but it was again not identified in *D. hansenii*'s genome (Table 6).

Finally, several membrane transport-related genes were induced in *S. cerevisiae*, that is *FET3* (iron transport), *PUT4* (aa transport) or *HXT1/5* (hexose transport) (Yale and Bohnert 2001). Up-regulation was also observed in our data set for *FET3* and *HXT5* in both conditions studied (high sodium or potassium), while *PUT4* was only over-expressed in the presence of NaCl. Interestingly, *HXT1* was slightly down-regulated in our study in the presence of NaCl. However, it appears to be up-regulated in *S. cerevisiae* according to some previous studies in the model yeast (Table 6).

Other examples of genes frequently found as repressed under salt stress in *S. cerevisiae* are *RRS1*

Table 6. Some of the significant genes found in literature to be important in the salt stress response and adaptation in *Saccharomyces cerevisiae*, and its role in *D. hansenii*. Information about the corresponding gene product was obtained from GRYC (Genome Resources for Yeast Chromosomes) database at iGenolevures (GRYC - Home page (inra.fr)) and UniProt database at (UniProt).

Protein	Function	GeneID (Sc)	GeneID (Dh)	Responsive to	
				NaCl	KCl
CTT1	Catalase T. Found in aerobically respiring organisms and serves to protect cells from the toxic effects of hydrogen peroxide	YGR088W	DEHA2B16214g	NO	NO
MSN4	Zinc finger protein. Positive transcriptional factor that acts as a component of the stress-responsive system (heat, oxidative, osmotic, etc.)	YKL062W	DEHA2A08382g	NO	NO
HLR1	Involved in cell wall composition and integrity and response to osmotic stress	YDR528W	–	–	–
GPD1	Glycerol-3-phosphate dehydrogenase 1. Catalyses the production and accumulation of glycerol during hyperosmotic stress conditions	YDL022W	DEHA2F09372g	YES	YES
GPD2	Glycerol-3-phosphate dehydrogenase 2. Catalyses the production of glycerol under anaerobic growth conditions	YOL059W	–	–	–
GPP1	Glycerol-1-phosphate phosphohydrolase 1 involved in glycerol biosynthesis. Plays a role in osmoadaptation in anaerobic conditions	YIL053W	DEHA2E16346g	YES	NO
GPP2	Glycerol-1-phosphate phosphohydrolase 2 involved in glycerol biosynthesis. Plays a role in osmoadaptation and it is induced in response to hyperosmotic or oxidative stress	YER062C	–	–	–
GLK1	Glucokinase-1. Catalyses the phosphorylation of glucose at C6 in the first irreversible step of glucose metabolism	YCL040W	DEHA2E06556g	NO	NO
HXK1	Hexokinase-1. Catalyses the phosphorylation of hexose, such as D-glucose and D-fructose, to hexose 6-phosphate	YFR053C	–	–	–
TPS1	Alpha-trehalose-phosphate synthase. Catalyses the production of trehalose from glucose-6-phosphate	YBR126C	DEHA2E21956g	NO	NO
TPS2	Trehalose-phosphatase. Phosphatase catalytic subunit of the trehalose synthase complex that catalyses the production of trehalose from glucose-6-phosphate	YDR074W	DEHA2G12452g	NO	NO
MET6	Cobalamin-independent methionine synthase. Catalyses the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine resulting in methionine formation	YER091C	DEHA2A07414g	NO	NO
MET25	Homocysteine/cysteine synthase. Catalyses the conversion of O-acetyl-L-homoserine (OAH) into homocysteine in the methionine biosynthesis pathway	YLR303W	DEHA2F06094g	NO	NO
CUP1-1	Copper metallothionein 1-1. Protects the cell against copper toxicity by tightly chelating copper ions	YHR053C	–	–	–
CUP1-2	Copper metallothionein 1-2. Protects the cell against copper toxicity by tightly chelating copper ions	YHR055C	–	–	–
FET3	Iron transport multicopper oxidase. Essential component of copper-dependent iron transport	YMR058W	DEHA2G05082g	YES	YES
PUT4	Proline-specific permease. Required for high-affinity proline transport. Also functions as non-specific GABA permease. Can also transport alanine and glycine	YOR348C	DEHA2B01078g	YES	NO
HXT1	Low-affinity glucose transporter. HXT1 is as well involved in the transport of mannose	YHR094C	DEHA2E04224g	YES	NO
HXT5	Hexose transporter with moderate affinity for glucose	YHR096C	DEHA2D18876g	YES	YES
RRS1	Regulator of ribosome biosynthesis	YOR294W	DEHA2F09834g	NO	NO
BFR2	Involved in endoplasmic reticulum to Golgi transport	YDR299W	DEHA2A14212g	NO	NO
HSP30	Negative regulator of the H+-ATPase Pma1p. It may counteract the altering effect of heat shock on the plasma membrane	YCR021C	DEHA2D05654g	NO	YES

—, The corresponding gene is not found in *D. hansenii*.

(ribosome biosynthesis), *BFR2* (ER to Golgi transport) and *HSP30* (heat shock protein) (Dhar *et al*., 2011). Neither *RRS1* nor *BFR2* was found as significantly responsive genes in the presence of NaCl or KCl in our analysis. On the other hand, *HSP30* expression showed to be repressed in *D. hansenii*, however only in the presence of KCl (Table 6).

Discussion

The main novelty of our study is the analysis, for the first time, of several -omics together under comparable conditions. The results obtained from both the transcriptomic and proteomic analyses suggest that more changes are always carried out in the presence of KCl (expression and protein abundance), compared with NaCl. This suggests a more specific and targeted response under sodium, while a less coordinated response seems to take place in the presence of potassium. Therefore, NaCl can be considered as a preferred salt for *D. hansenii* to grow, as the authors already reported in a very recent work by characterizing this yeast in highly controlled and monitored bioreactors (Navarrete *et al*., 2021).

Two important aspects must be considered in this study. On the one hand, the fact that *D. hansenii* cells

were already adapted to the high salt environmental conditions at the time of the sampling. Cells growing in continuous cultivation, once steady state has been reached, present as advantage the certainty that the changes observed compared with the control conditions (no salt present) are exclusively due to the presence of high salt, since all the other factors remain constant (temperature, pH, substrate concentration, biomass concentration, growth rate, etc). But at the same time, transient and fast changes (what we can define as 'fast' or 'immediate' response) are probably missed. On the other hand, it is important to emphasize that the salt concentration (1 M) used in this study is the optimal for *D. hansenii*'s performance, according to our most recent publication on the physiology of this yeast (Navarrete *et al*., 2021). This can explain why some genes/proteins typically related to stress response in yeast were nevertheless not found relevant in the present study.

The list of differentially expressed genes and proteins with changes in their abundance presented in this work (Tables 1–2 and S1-S4) led us to some general observations regarding the metabolic changes experienced by *D. hansenii* under high salt conditions. From both types of analyses, RNA-seq and global proteomics, and in the presence of NaCl or KCl, an increase in the respiratory metabolism is observed, which aligns with previous reports by Navarrete *et al*. (2021) and García-Neto *et al*. (2017). Enhanced glucose (hexoses) uptake, phosphate uptake, zinc transport or iron uptake was observed and is well known to be directly related to an increased respiratory metabolism and therefore to a higher growth and better performance of the cells under these conditions.

Another important aspect related to the salt tolerance in *D. hansenii* is the role of different transmembrane transport systems in this response. In our work, this is confirmed by the over-expression of *ENA1* and *KHA1* genes (Na^+-ATPase and Na^+/H^+ antiporter in the Golgi apparatus respectively), as well as by an increase in protein abundance, also for Ena1 and Vma22 (H^+-ATPase in the vacuole), among others. Interestingly, and as mentioned before, the over-expression of genes involved in phosphate uptake (e.g. *PHO84*) is directly related to a higher metabolic rate. The more the cells grow, the more the medium gets acidified; therefore, P^+ transport inside the cell is favoured at the same time the growth capacity is stimulated. This effect is higher in the presence of NaCl, due to a faster growth in these conditions compared with KCl, and also because the expression of *PHO84*, but specially *PHO89*, is significantly higher in the presence of sodium compared with potassium (Fig. 5). In a previous study, Sanchez *et al*. (2008) reported an increased intracellular phosphate levels upon exposure to either 1 M KCl or NaCl, being the accumulated intracellular phosphate high in the presence of both salts compared with no salt added, however higher in the presence of sodium compared with potassium, which agrees with our observations, proving that it is caused by the increased over-expression of the *PHO89* Na^+/phosphate co-transporter. Changes in the expression of other transporters such as Trk1 or Hak1 are not observed at any level, most probably because the cells are already adapted to the conditions studied (chemostats) and fast-response changes are not expected to be detected under our analysis, as mentioned earlier. Glycerol synthesis and uptake are also increased in the presence of both salts, and glycerol has been previously reported to play an important role in the maintenance of osmotic pressure under these conditions (Prista *et al*., 2016; Sanchez *et al*., 2020). In this regard, our data show that an additional glycerol-3-phosphatase gene (*RHR2*) is over-expressed besides *GPP1*, thus significantly contributing to the glycerol biosynthesis in high salt concentrations.

The oxidative stress response is also stimulated by the presence of both NaCl and KCl. Several proteins related to the cell response to this type of stress are more abundant or increased their translation (e.g. Sod1, Ctt1 and Srx1). Several authors had already described the protective effect of salt (especially sodium) against oxidative stress damage in *D. hansenii* (Navarrete *et al*., 2009; Ramos-Moreno *et al*., 2019). Again, this can be linked to a higher respiratory metabolism stimulated by the presence of salt, leading to an accumulation of intracellular ROS (reactive oxygen species) triggering the activation of this response. Strikingly, there is also an associated increase in the resistance to other drugs caused by high sodium levels, as over-expression of genes involved in drug resistance was observed (i.e. *TPO1* and *PDR5*); some of them are also related to cation resistance in *S. cerevisiae* (Yale and Bohnert 2001; Teixeira and Sá-Correia 2002). This can be of high relevance if *D. hansenii* is used for growth in complex biomass hydrolysates as feedstock, in industrial fermentation setups, which are usually rich in the presence of inhibitory compounds as a consequence of the pretreatments for releasing fermentable sugars (Navarrete *et al*., 2020).

Alone from the proteomic analysis, and in the presence of both salts, some important conclusions can be made. First, secondary carbon source utilization is stimulated (pentoses catabolism), as arabinose, maltose and xylose metabolism-related proteins are over-represented in our study (Tables S3 and S4). This is of great importance for the potential industrial use of *D. hansenii*, as the capacity of metabolizing a wide array of different carbons is a beneficial (and demanded) trait for microbial cell factories. In addition, proteins related to the cell membrane and cell wall integrity are also over-

represented; that is cell wall protein precursors and assembly-related proteins or oligopeptide transporters related to the maintenance of lipid asymmetry in the membrane (e.g. Gas1, Cwp1, Opt2, Cts2, Chs3, Stt4 and Ole1) (Tables S3 and S4). Changes in the membrane composition and fluidity, as well as in the cell wall structure, had been previously suggested by Turk *et al*. (2007), Michán *et al*. (2013) and Kodedová and Sychrová (2015), so our results confirm their findings and add relevant information about the protein targets implicated in the described processes. The subsequent phosphoproteomics analysis also revealed important key enzymes involved in fatty acids biosynthesis (Q6BII7 and Q6BWN1) and cell wall biosynthesis and integrity (Q6BJY4 and Q6BYG3), being highly phosphorylated (hence significantly activated) in response to high sodium concentrations. (Table 4).

Due to the differences already reported by previous studies in *D. hansenii* in response to either sodium or potassium, changes in expression related to each specific one were also expected in our analysis. A good example of this is the fact that, in the presence of NaCl, a repression of genes involved in the aromatic amino acid biosynthesis was found (Table S1). Interestingly, an increase in the abundance of proteins related to translation processes is generally observed at a proteomic level (Table S3). This could be explained because of a high protein turnover in the cells, ensuring a sufficient amount of free amino acids to synthesize proteins without increasing the expression of the genes involved in *de novo* amino acid biosynthesis. Sporulation mechanisms are also repressed in the presence of NaCl, at both transcriptomic and proteomic levels (Tables S1 and S3). This can relate to the increased growth observed in these conditions: if cell performance is stimulated by the presence of sodium, there is no need to increase the expression of genes involved in spore formation.

On the other hand, regarding responses exclusively caused by the presence of KCl, very clearly both riboflavin and lipid metabolism are boosted. Both biosynthetic processes are enhanced at a transcriptomic and proteomic level under this condition (Tables S2 and S4). This can have significant relevance, and it is a very important factor to consider in industry, if *D. hansenii* is used to produce lipid-derived compounds or antioxidants for example. Besides, the proteomic analysis revealed a decrease in processes like the amino acid biosynthesis and high-affinity glucose transport, when KCl is present in the culture media. The latter could explain why the growth rate, and more specifically the glucose uptake rate, is higher in the presence of sodium over potassium (Navarrete *et al*., 2021).

Two important aspects need to be considered in future investigations. On one hand, it is of extreme importance to dedicate a stronger research effort to investigate those genes/proteins with unknown function or yet uncharacterized that were found in the present study. A significant fraction of the most relevantly expressed/repressed genes in the presence of either high sodium or potassium still remains unknown, which hampers the full elucidation of the molecular mechanisms that lead to the high halotolerance/halophilicity of *D. hansenii*. A suggested strategy could be the generation of knock-out mutants for those unknown genes that are specially over-expressed and investigate their possible role in *D. hansenii*'s metabolism. At a protein level, the investigation could be focussed on the study and identification of motifs/regions that could help us to understand their function or to include them in an already existing category. Still to date, there is a lack of proper editing mechanisms to precisely engineer this peculiar yeast, and although some very recent advances have been made in this area (Spasskaya e*t al*., 2021), the development of efficient genetic tools of high precision for *Debaryomyces* is still in its infancy.

What is certainly the most innovative aspect of our work is that it contains a phosphoproteomic study on the response to high salt, which is the first time ever reported of this kind for *D. hansenii*. The results of the analysis revealed interesting information to better understand the mechanisms of salt tolerance in this peculiar yeast. Very interestingly, the metabolic response to sodium seems to be highly coordinated and more regulated in comparison with the response to the presence of high potassium concentrations in the environment, which is evidenced by the amount of responsive proteins involved (36 proteins showed to be highly phosphorylated with sodium vs. only 4 in response to potassium). The analysis also revealed processes never before linked to the halotolerance response, implicating mechanisms like protein trafficking, endocytosis and biosynthesis of long fatty acids. Another relevant process such as targeted protein degradation had previously been reported as an important player in salt tolerance in *D. hansenii* (Spasskaya *et al*., 2021), and our study confirms its relevance as, apart from several genes identified in the general transcriptomic and proteomic analyses, the phosphoproteomic analysis showed three of the key regulatory enzymes to be involved in ubiquitin-mediated degradation (Q6BRB3, Q6BU81 and Q6BUM7) (Table 4). Strikingly, we found the existence of an uncharacterized cation transmembrane transporter unknown to date in *D. hansenii* (B5RUG0), highly phosphorylated in response to high sodium, which shows homology with a cation transporter present in some *Candida* species and involved in metal tolerance (Table 4). This transporter could be a very relevant key factor for halotolerance never considered before; therefore,

advancing in its study and characterization could be paramount to better understand halotolerance in this yeast.

Overall, probably the most relevant conclusion to be made from the phosphoproteomic data analysis is that the response to sodium seems more coordinated and involves a higher level of regulation of protein activity, while the response triggered by the presence of potassium seems vaster and barely regulated compared with sodium. This was initially anticipated by the number of genes/proteins showing a differential expression upon exposure to sodium vs. potassium, and the PCA analysis of the sample population, and finally confirmed by the phosphoproteomic analysis. Along the same line, it is also important to mention that the genes coding for the phosphate transporters *PHO84* and *PHO89* were among the top-15 over-expressed with the higher significance in the presence of sodium (Table 1), while their expression in the presence of potassium, although also stimulated, occurred at much lower levels (specially *PHO84*), reinforcing the importance of the coordinated and highly regulated response through control of enzyme activity (via phosphorylation) in response of sodium.

A worth mentioning limitation that we found during this work was the lack of GEMs (genome-scale models) available for *D. hansenii*, which would have significantly contributed to a better understanding of the metabolic fluxes in this yeast, under the presence of both NaCl and KCl. By the followed approach, we could only point out to mechanisms or processes that seem to be directly related to the response and adaptation to high salt: the most evident ones. However, we could not make conclusive assertions about how they would ultimately affect *D. hansenii*'s metabolism as a whole.

Experimental procedures

Strain and culture conditions

The *D. hansenii* strain CBS 767 (PYCC2968; Prista *et al*., 1997; Navarrete *et al*., 2009, 2020) was used in this study. The strain was stored at −80°C in glycerol stocks containing sterile 30% glycerol (Sigma-Aldrich, Germany).

Cells were grown from the cryostocks at 28°C in Yeast extract Peptone Dextrose (YPD) medium plates with 2% agar. For the pre-cultures of yeast cells, synthetic complete medium (YNB) was used (6.7 g l^{-1} Yeast Nitrogen Base w/o amino acids, from Difco, plus 0.79 g l^{-1} complete supplement mixture, from Formedium). Separately sterilized 2% D-(+)-glucose monohydrated (VWR Chemicals, VWR International, Darmstadt, Germany) was added to the medium, and the pH was adjusted to 6.0 with NaOH. All the solutions were autoclaved at 121°C for 20 min. Pre-cultures of 100 ml cell culture were incubated in 500 ml baffled Erlenmeyer shake flasks at 28°C, 150 rpm for at least 24 h.

Bioreactor cultivations

Batch cultivations were first run for about 72 h in biological replicates (between 3 and 5 per condition) in 1.0 l Biostat Qplus bioreactors (Sartorius Stedim Biotech, Göttingen, Germany). The temperature was controlled at 28°C, and pH was maintained at 6.0 by the automatic addition of 2 M NaOH/2 M H_2SO_4 and measured by pH sensors (Model EasyFerm Plus K8 160; Hamilton, Bonaduz GR, Switzerland). The volumetric flow rate (aeration) was set at 1 vvm, and the stirring was constant at 600 rpm. The working volume in the vessel was 0.5 l, using exactly the same medium composition as in the pre-culture, and containing 2% of glucose. When needed, 1 M NaCl or KCl (PanReac Applichem, ITW Reagents) was added to the medium. The bioreactors were inoculated with 24 h inoculum from the pre-culture to get an initial OD_{600} of 0.05–0.1.

Once the carbon source was completely depleted, the feeding was started at a dilution rate of 0.1 h^{-1}. The chemostat was run with a carbon limitation of 1% of glucose, and 1 M NaCl or KCl was added to the feeding bottle when needed. Sampling took place after 5 residence times (≈ 50 h).

Sampling from bioreactors

Figure 7 shows a simplified scheme of the experimental design followed in this work. The sampling procedure was slightly different depending on the type of samples required. Nevertheless, it was always performed at 4°C.

Cells taken for transcriptomics analysis were centrifuged at 5000 g for 5 min. The supernatant was discarded, and the pellets were immediately frozen in liquid nitrogen and stored at −80°C. The RNA from the stored biomass samples was extracted using the RNeasy Mini Kit from Qiagen according to the user´s manual (Qiagen, Hilden, Germany). RNA concentration and integrity of the extracted samples were determined by Nanodrop Lite (Thermo Scientific, Waltham, MA, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively.

Samples for proteomics analysis were also centrifuged at 5000 g for 5 min. The supernatant was discarded, and the pellets were washed with 20 ml of ice-cold 1x PBS buffer (8 g l^{-1} NaCl, 0.2 g l^{-1} KCl, 1.44 g l^{-1} Na_2PO_4 and 0.24 g l^{-1} KH_2PO_4, pH 7.4 adjusted with HCl). Cells were centrifuged again (5000 g, 5 min), and pellets were frozen at −20°C until further use. The stored biomass samples were thawed on ice, and 500 μl of thawed pellet was mixed with 1 ml of lysis buffer (6 M guanidinium

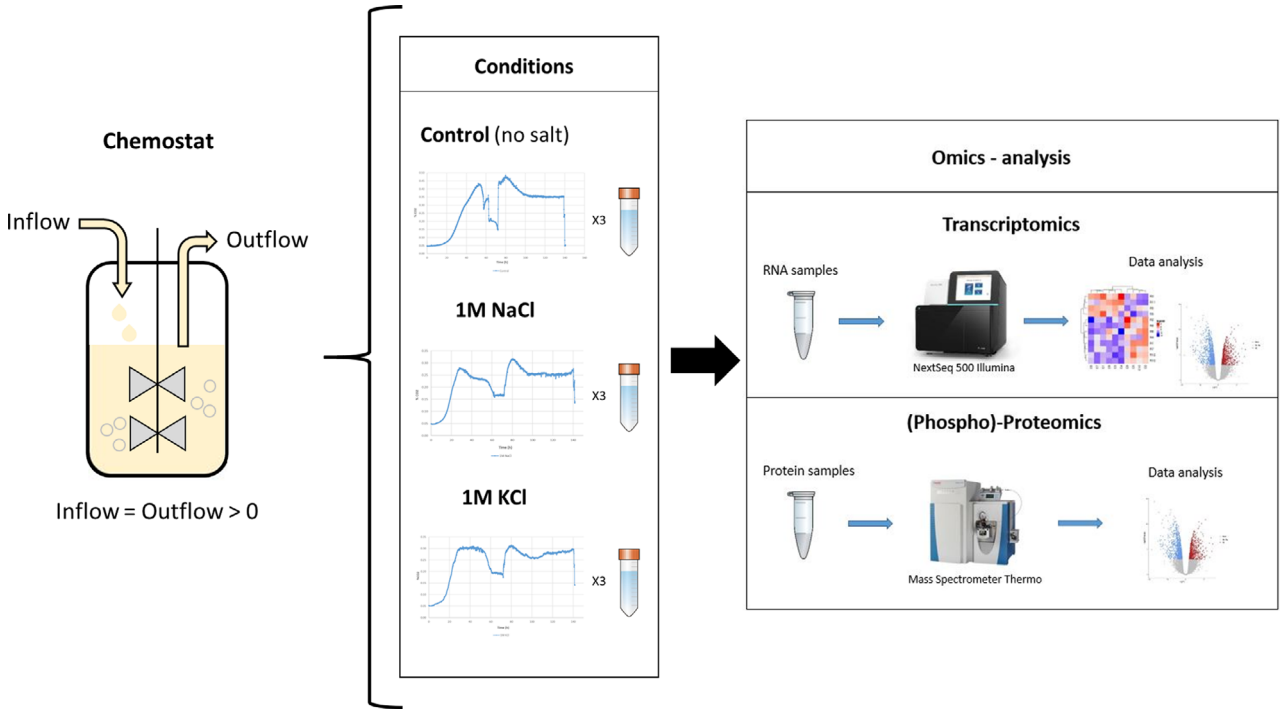

Fig. 7. Experimental design that shows the bioreactor system used, conditions tested and -omics studied in this work.

hydrochloride, 10 mM Tris(2-carboxyethyl)phosphine hydrochloride, 40 mM 2-Chloroacetamide and 50 mM HEPES pH 8,5, all from Sigma-Aldrich, Darmstadt, Germany). After briefly mixing by vortex, the samples were boiled at 95°C for 5 min in a block heater, vortex again and then cooled on ice. One millilitre of the cooled samples was transferred to ice-cold 2 ml screw-capped tubes along with ≈ 400–500 µl of 0.5 mm sterile glass beads for 3 × 20 s cycle at speed 6,5 in a FP120 FastPrep® homogenizer cell disrupter (Thermo Savant, France). Samples were cooled on ice for one minute between cycles. After disruption, tubes were spun down for 15 min at 15 000 g and 4°C, and the supernatant transferred to clean tubes and stored at −20°C. The protein concentration was determined by the Bradford´s method using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and according to the user´s manual. Finally, proteins were handed over to the Proteomics Core (DTU Bioengineering, Kgs Lyngby, Denmark), where they performed a treatment with tripsin (1:100 enzyme to protein ratio) and run the samples in the MS instrument according to their standard protocols.

The phosphoproteomics data were obtained from the same protein extractions as the samples for global proteomics analysis, but in this case, the samples were phospho-enriched at the Proteomics Core (DTU Bioengineering) and run separately in the MS instrument according to their specific protocol.

RNA-seq data analysis

RNA library preparation and sequencing were performed at the NGS Core service at DTU Biosustain (https://www.biosustain.dtu.dk/innovation/translational-core), using a NextSeq 500 sequencing system from Illumina (Illumina, San Diego, CA, USA).

RNA-seq raw data were analysed/visualized by using Galaxy (https://usegalaxy.org/), an open-source web-based platform for multi -omics analysis. Quality control on raw data coming from the sequencing pipelines was performed by FastQC (Babraham Bioinformatics) and visualized by MultiQC (Ewels *et al*., 2016).

RNA STAR (Dobin *et al*., 2013) was used to perform the alignment of the RNA-seq data to our *D. hansenii* CBS767 reference genome [obtained from Pubmed genome database; *Debaryomyces hansenii* (ID 195) – Genome – NCBI (nih.gov)]. The mapping percentage was between 93% and 97% and the uniquely mapped reads varied between 14.7 and 18.4 millions, depending on the sample.

featureCounts (Liao and Smyth, 2014) was used to obtain the counts reads mapped per gene in our annotated reference genome (also downloaded from Pubmed genome database), in order to measure the gene expression in our experiments. Between 27.8 and 35 millions of reads were assigned per sample, with a percentage of assignment between 84.6% and 90.5%. Finally, edgeR (Robinson and McCarthy, 2010) was

used to normalize the data and perform the differential gene expression analysis.

Gene enrichment analysis

A gene enrichment analysis of the RNA counts was performed using goseq (Young *et al*., 2010), also available at the Galaxy platform. This tool performs gene enrichment considering length bias, and it was used for finding both GO terms and KEGG pathways that show a significantly different number of genes either over-expressed or under-expressed. To consider a group (GO:term or KEGG pathway) as significant, we filtered for those composed by at least five genes and with a FDR < 0.05.

Information about each identifier for *D. hansenii* was obtained from the Gene Ontology and KEGG websites (http://geneontology.org/ and KEGG: Kyoto Encyclopedia of Genes and Genomes).

Global proteome and phosphoproteome analysis

The global proteome and phosphoproteome raw data, from mass spectrometry analysis, were directly provided by the Proteomics Core at DTU Bioengineering (DTU Proteomics Core – DTU Bioengineering). The equipment used was a Exploris 480 Orbitrap MS operated in Data Independent Acquisition mode coupled to Evosep One instrument running 30SPD method, and data were searched using Spectronaut 11 software against *D. hansenii* CBS767 reference proteome. Phosphoproteomics search was set to identify and quantify phospho (Ser and Tyr) modifications.

The differential analysis of the normalized total ion count (global proteomics and phosphoproteomics) was performed using edgeR (Robinson *et al*., 2010) in the same way as for the RNA-seq data, in order to get the differential protein abundance for each sample.

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Author's contributions

JLM conceived the project. CN designed and performed the experiments. CN, BJS and SS analysed the data. CN and JLM wrote the manuscript. All authors read, edited and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Percentage of genes (ORFs) with (A) logFC>2 or (B) logFC<−2 identified from the differential analysis of the RNA-seq data (FDR<0.05). Dark bars represent significant genes obtained in NaCl, whereas light bars represent significant genes obtained in KCl.

Fig. S2. Percentage of proteins and genes (ORFs) of unknown function obtained from the differential analysis (−2>logFC>2, FDR<0.05). Dark bars represent NaCl, whereas light bars represent KCl.

Fig. S3. Bars diagram representing GO:terms obtained from the enrichment analysis of RNA-seq data in (A) NaCl or (B) KCl. Orange bars represent the number of differentially expressed (DE) ORFs in each category. Blue bars represent the number of ORFs in each category from the total

pool analysed. All the categories selected for these diagrams showed a FDR<0.05 in the analysis.

Fig. S4. Bars diagram representing KEGG pathways obtained from the enrichment analysis of RNA-seq data in (A) NaCl or (B) KCl. Orange bars represent the number of differentially expressed (DE) ORFs in each category. Blue bars represent the number of ORFs in each category from the total pool analysed. All the categories selected for these diagrams showed a FDR<0.05 in the analysis.

Fig. S5. Bars diagram representing KEGG modules obtained from the enrichment analysis of RNA-seq data in (A) NaCl or (B) KCl. Orange bars represent the number of differentially expressed (DE) ORFs in each category. Blue bars represent the number of ORFs in each category from the total pool analysed. All the categories selected for these diagrams showed a FDR<0.05 in the analysis.

Fig. S6. Percentage of proteins with (A) logFC>2 or (B) logFC<−2 identified from the differential analysis of the global proteomic data (FDR<0.05). Dark bars represent significant proteins obtained in NaCl, whereas light bars represent significant proteins obtained in KCl.

Table S1. Significant *D. hansenii*'s gene products with a -2>logFC>2 in the presence of NaCl. The identified gene ID is colored in red when up-regulated or in blue when down-regulated. Information about the corresponding gene product was obtained from GRYC (Genome Resources for Yeast Chromosomes) database at iGenolevures [GRYC - Home page (inra.fr)].

Table S2. Significant *D. hansenii*'s gene products with a -2>logFC>2 in the presence of KCl. The identified gene ID is colored in red when up-regulated or in blue when down-regulated. Information about the corresponding gene product was obtained from GRYC (Genome Resources for Yeast Chromosomes) database at iGenolevures [GRYC - Home page (inra.fr)].

Table S3. Significant *D. hansenii*'s proteins with a -2>logFC>2 in the presence of NaCl. The identified protein ID is colored in red when up-represented or in blue when down-represented. Information about the corresponding protein was obtained from UniProt database at (UniProt).

Table S4. Significant *D. hansenii*'s proteins with a -2>logFC>2 in the presence of KCl. The identified protein ID is colored in red when up-represented or in blue when down-represented. Information about the corresponding protein was obtained from UniProt database at (UniProt).

