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

Clara Navarrete, Benjamin J. Sánchez, Simonas Savickas and José L. Martínez*  
Department of Biotechnology and Biomedicine, Technical University of Denmark, Solttofs Plads Building 223, Kgs. Lyngby, 2800, Denmark.

**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.

**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.
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 H₂O₂, 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 (Waite et al., 2001).

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 (Martinez and Luna, 2012). In this work, the authors identified up-regulated 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 Šychrova 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
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 $-2<\log_{10}\text{FC}<2$ (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 $2<\log_{10}\text{FC}<5$ and only 11.76% (NaCl) and 8.47% (KCl) present $\log_{10}\text{FC}>5$ (Fig. S1A). Instead, the 95.83% (NaCl) and 100% (KCl) of the down-regulated genes show a $-2<\log_{10}\text{FC}<5$ (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 $-2<\log_{10}\text{FC}<2$ (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:000750) 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...
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. 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).
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.
when potassium is present (Fig. 4B). (dhaM00158) modules are significantly up-regulated when sodium is present, whilst ergocalciferol biosynthesis (dhaM00102) and F-type ATPase (dhaM00158) modules are significantly up-regulated when potassium is present (Fig. 4B).

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 (rina.fr)).

| Gene ID       | Gene product                                                                 |
|---------------|-----------------------------------------------------------------------------|
| DEHA2E14234g  | PHO84 high-affinity inorganic phosphate/H+ symporter                        |
| DEHA2E3958g   | 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  | 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  | AR03 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                                                             |

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.

| 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 (rina.fr)).

| Gene ID       | Gene product                                                                 |
|---------------|-----------------------------------------------------------------------------|
| DEHA2E3958g   | 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  | AR03 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                                       |

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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 (Q6BT15), 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 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
â–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, downregulated GO:terms or KEGG pathways were more abundant than up-regulated ones.

**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, Q6BK7 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)]DFEIPDIDYGDDS[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 salinity.

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**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 / KEGG pathway                      |
|-----------|--------------------------------------------|
| 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)           |
|           | Oxidoreductase activity (GO:0016491)       |
|           | Nucleoside metabolic process (GO:0009116)   |
|           | Intracellular protein transport (GO:0006886) |
|           | Ran GTPase binding (GO:0005836)            |
| 1 M KCl   | Membrane (GO:0016020)                      |
|           | Biosynthesis of unsaturated fatty acids (dha01040) |

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sodium. None of the previous studies on *D. hansenii* 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 osmo-regulation 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_ | RFX1. rRNA processing |
| Q6BNR1     | _NINNP[Phospho (STY)]DLEEAAR_ | I-B22. Mitochondrial electron transport |
| B5RST1     | _GASDDOSILS[Phospho (STY)]LDEK_ | P3C domain containing. Glycerolipid metabolism |
| Q6RBR3     | _DSS[Phospho (STY)]PTAPTSTSTSTSGATSVSNSWAAALK_ | DEF1. RNA polymerase II degradation factor 1 |
| Q6BWR2     | _EQU[Phospho (STY)]PDSTILVSK_ | Uncharacterized protein |
| Q6BU81     | _ADEEEDE[Phospho (STY)]DFVADENNDENDK_ | Ubiquitinyl hydroxylase 1 |
| Q6BV47     | _AELES[Phospho (STY)]LIQ_ | SAR1. Transport vesicles from the ER |
| Q6BU46     | _VGA66[Phospho (STY)]EQQFPQPFALFK_ | DED81. Cytosolic asparaginyl-RNA synthetase |
| Q6BUK3     | _YQFENDS[Phospho (STY)]JEDDEMEK_ | SEC9. t-Snare protein |
| B5RT37     | _IFNASSNS[Phospho (STY)]LSSM[Oxidation (M)]SGK_ | Uncharacterized protein |
| Q6BK57     | _GPDAAEGFADAVDELLOS[Phospho (STY)]NQLDTK_ | Uncharacterized protein |
| Q6BH11     | _SSIDDEE[Phospho (STY)]TEETEEPAK_ | Uncharacterized protein |
| Q6BW98     | _IDDDDVYS[Phospho (STY)]EIDK_ | Uncharacterized protein |
| Q6BI94     | _EPEPADDLEYES[Phospho (STY)]MDEAECA[Carbamidomethyl (C)]A_ | Uncharacterized protein |
| B5RT82     | _NIT[Phospho (STY)]PNASNDDSLV_ | Arf-GAP. ER to Golgi vesicle-mediated transport |
| Q6BJU4     | _SQOPLINM[Oxidation (M)]ESS[Phospho (STY)]K_ | SM11. Regulation of cell wall biosynthesis |
| Q6BV91     | _YTSNNLVNDPSG[Phospho (STY)]DDERGRER_ | PRP45. Pre-mRNA-processing protein 45 |
| Q6BRE1     | _PLG[Phospho (STY)]TSALNK_ | Uncharacterized protein |
| Q6BZ08     | _EVSEVNLAS[Phospho (STY)]ITSLH[Phospho (STY)]VPS[Phospho (STY)]JAVK_ | Uncharacterized protein |
| Q6BU7M     | _S[Phospho (STY)]SLFSRDNVTVN_ | Non-specific serine/threonine kinase |
| B5RTL6     | _FS[Phospho (STY)]IGNSLGQSR_ | Elongation of fatty acids related-protein |
| Q6BI7     | _S[Phospho (STY)]DTPVPEAQIR_ | Uncharacterized protein |
| Q6BI8     | _VQDVGPSNTRENDYAVAS[Phospho (STY)]NK_ | Non-specific serine/threonine kinase |
| Q6BPF2     | _EVEK_ | Uncharacterized protein |
| B5RUF3     | _LSSEARDES[Phospho (STY)]EIS[Phospho (STY)]ALENVTNDIK_ | PWWP protein. Histone methylation |
| Q6BKZ0     | _DDLPSOSQEYESVINS[Phospho (STY)]ESESVR_ | Uncharacterized protein |
| B5RT30     | _DENNL[Phospho (STY)]DEWSMD[Phospho (STY)]VHLK_ | Oxidoreductase. FAD-binding protein |
| Q6BW31     | _TINS[Phospho (STY)]SNLHLIK_ | Transmembrane transporter activity |
| Q5RUG0     | _SPS[Phospho (STY)]DSQFASVTVN_ | Cation transmembrane transporter activity |
| Q6BX9     | _QT[Phospho (STY)]VTPVIVQIR_ | Uncharacterized protein |
| Q6BGQ1     | _SLF[Phospho (STY)]TPNTSTTSTSGATSVSNSWAAALK_ | 3-isopropylmalate dehydratase |
| Q6BWN1     | _PS[Phospho (STY)]NPLASH[Phospho (STY)]EDDEIEAS[Phospho (STY)]EDIEAS[Phospho (STY)]TPD_ | Uncharacterized protein |
| Q6BUD7     | _QSTDDS[Phospho (STY)]DMQNDAPPOEAK_ | Uncharacterized protein |
| Q6BYG3     | _APY[Phospho (STY)]PPPFTVFQ_ | Uncharacterized protein |
| Q6BV90     | _DIDSNS[Phospho (STY)]EEEDEEEEDEENELVGR_ | WAC domain containing protein. Cell growth |
| B5RTY0     | _E[C][Carbamidomethyl (C)]IDVPALHS[Phospho (STY)]DQOSR_ | 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)]EVEEAK_ | TRR1 thioredoxin reductase |
| Q6BJK1     | _ATE[Phospho (STY)]NVEEOEDDEEI EEDDDAFHKK_ | ATPase activity. Ribosome biogenesis |
| Q6BHH7     | _NIN[Phospho (STY)]PPK_ | RMM. RNA recognition motif |
| Q6BUY1     | _DDAESEY[Phospho (STY)]ET [Phospho (STY)]DGEI_ | Uncharacterized protein |

been described in several studies for this purpose, and as an example, genes involved in ion homeostasis and osmolites 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
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, HKX1, 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 HKX1 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) | 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 | DEHA2E00556g | NO | NO |
| HXX1 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 | Catalyses the 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 | DEHA2B00227g | NO | NO |
| HXT1 Low-affinity glucose transporter. HXT1 is as well involved in the transfer of mannose | | YHR094C | DEHA2E04224g | YES | YES |
| 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 transcripomic 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 Garcia-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 arabinoose, 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), Michan 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 (Q6BlI7 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 spor 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 et 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, WVR 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 H₂SO₄ 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 OD₆₀₀ 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 RNaseasy 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 Na₂PO₄ and 0.24 g l⁻¹ KH₂PO₄, 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
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 trypsin (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 Explotr 480 Orbitrap MS operated in Data Independent Acquisition mode coupled to Evoset One instrument running 30SPD method, and data were searched using Spectronaut 11 software against 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).