Identification and investigation of osmostress-induced genes in *Dictyostelium discoideum*

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Jianbo Na

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Prof. Dr. Angelika A. Noegel
Prof. Dr. Diethard Tautz

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Jianbo Na
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Cologne, Germany

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1 Introduction

1.1 *Dictyostelium discoideum* as a model organism

The social amoeba *D. discoideum* lives in deciduous forest soil and feeds on bacteria. During its life cycle, *D. discoideum* switches between unicellularity and multicellularity. During the unicellular phase, where cells are highly motile, *D. discoideum* is an excellent model for studying fundamental cellular processes, such as cytokinesis, phagocytosis, chemotaxis, and signal transduction (Noegel and Schleicher, 2000; Van Haastert and Devreotes, 2004). Upon depletion of the food source, *D. discoideum* cells signal to each other by cyclic AMP (cAMP) pulses. Signal sensing and signal response lead to streams of migrating cells, which then form an aggregate consisting of up to 100,000 cells. The aggregate further develops to a tipped mound, which either becomes a migrating slug or goes directly to culmination, the formation of a fruiting body (Chisholm and Firtel, 2004) (Figure 1). Therefore, *D. discoideum* is also a good model organism to study aspects of development such as cell sorting, pattern formation, and cell-type differentiation.

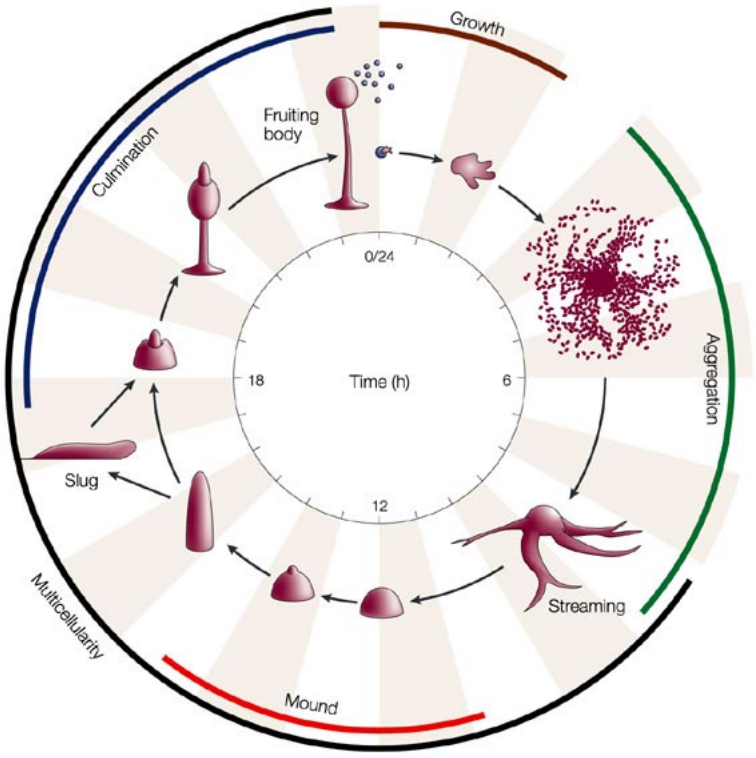

Figure 1. *D. discoideum* morphogenesis (Chisholm and Firtel, 2004).

The genome of *Dictyostelium* has a size of 34 Mb which are distributed over 6 chromosomes and encode approximately 12,500 genes. A proteome-based phylogenetic analysis placed *D. discoideum* prior to the split of metazoa and fungi but after the divergence of the plant kingdom (Eichinger et al., 2005) (Figure 2). It is therefore likely that research results obtained with *Dictyostelium* are also relevant to higher organisms, in particular metazoan. The *Dictyostelium* genome (Eichinger et al., 2005) and cDNA projects (Urushihara et al., 2004) paved the way for functional study of *Dictyostelium* at a genome wide scale.

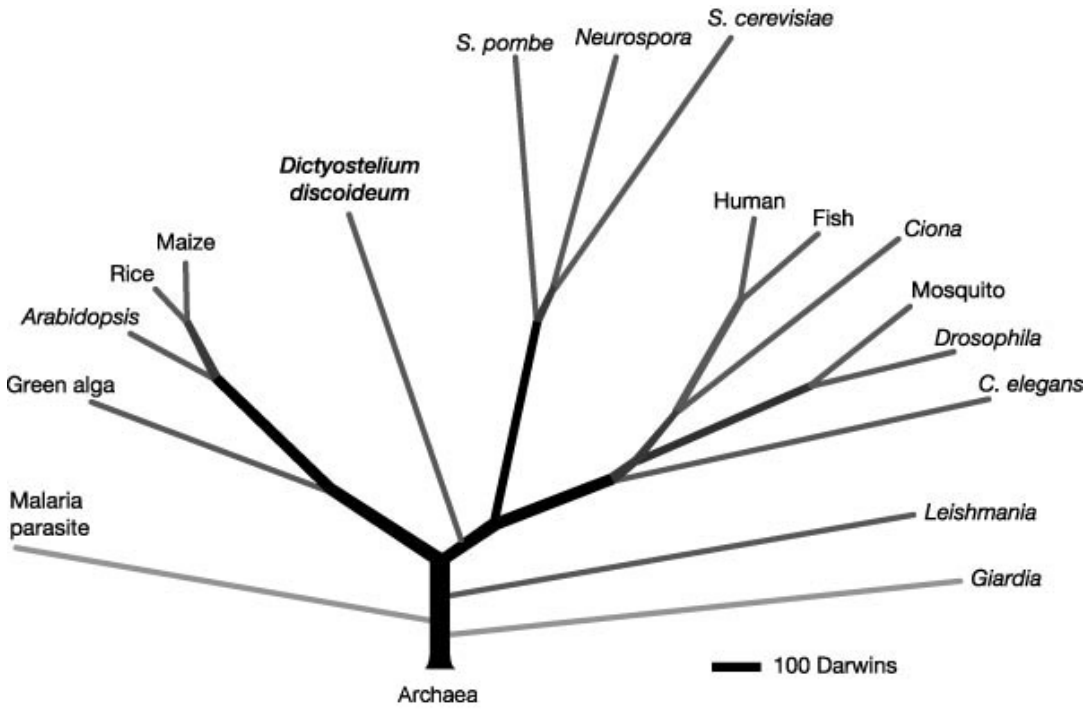

Figure 2. Proteome-based eukaryotic phylogeny (Eichinger et al., 2005).

1.2 DNA-Microarrays

The completion of the genomic sequences of many model organisms allows placing the emphasis on assigning functions to genes. One aspect of functional genomics is to determine the expression patterns of genes. Various methods are available for detecting and quantitating gene expression levels, including Northern blots (Alwine JC, 1977), S1 nuclease protection (Berk and Sharp, 1977), differential display (Liang and Pardee, 1992), sequencing of cDNA libraries (Adams et al., 1991; Okubo et

al., 1992), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), and DNA microarrays (Schena et al., 1995). Most of these methods are either limited to a small scale, difficult to process or inefficient on a large scale. In contrast, DNA microarrays are meanwhile mature tools to investigate differential expression of thousands of genes in parallel (Schena et al., 1995; Schena et al., 1996).

DNA microarrays consist of an orderly arrangement of probes of cDNA, genomic DNA or synthesized oligonucleotides that represent individual genes of an organism (Schulze and Downward, 2001). DNA microarrays are widely used in large-scale DNA mapping (Poustka, 1986), sequencing (Cantor et al., 1992) and transcript-level analyses (Schena et al., 1995). Recently, this technology has spread into many areas by adapting the basic concept and combining it with other techniques (Hoheisel, 2006). Among them cDNA microarrays are the most commonly used, and were first described in 1995 (Schena et al., 1995). Partial ORFs or cDNAs are amplified by PCR, purified and spotted on a glass slide. mRNA from the test and the reference cells are reverse transcribed to cDNA, and labelled with different fluorescent dyes. The fluorescent targets are pooled and hybridized under stringent conditions to the probes on the microarray glass slide simultaneously. The laser excitation of the targets yields an emission of specific spectra, which is measured using a scanning confocal laser microscope. Monochrome images from the scanner are imported into software in which the images are pseudo-coloured and merged. The signal intensity of each spot from both channels is quantitated, thus allowing the determination of the relative amount of transcripts (Duggan et al.) (Figure 3).

Since cDNA microarrays represent most or even all genes of an organism, they can be used to identify differentially regulated genes in any given experimental condition and outline the complete transcriptome picture. A good example is the application of cDNA microarrays in the classification of predefined cancer subtypes at the molecular level (Alizadeh et al., 2000; Golub et al., 1999; Simon et al., 2003). Another example is the use of microarrays to better understand the host response upon infection with pathogens (Cohen et al., 2000; Dowd et al., 2004; Farbrother et al., 2006). Besides, microarrays are also ideally suited to study the stress response and are widely applied to different organisms (Baxter et al., 2007; Hohmann, 2002; Jamers et al., 2006; O´Rourke et al., 2002).

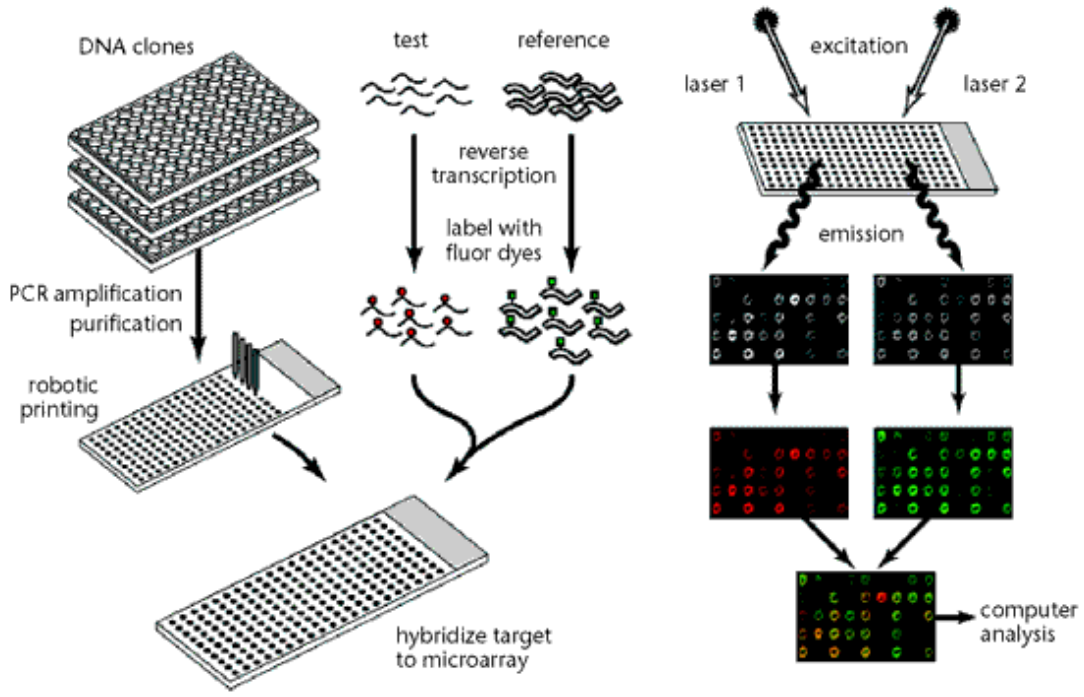

Figure 3. The principle of cDNA microarrays (Duggan et al., 1999).

1.3 Osmotic stress response

Virtually all cells, even individual cells in multi-cellular organisms, are subject to changes in the osmotic environment that are sometimes extremely rapid. In order to survive cells have to sense these changes and elicit an appropriate response that allows them to adapt. The response is complex and occurs in different phases. First, immediate cellular changes occur as a consequence of stress exposure, then defence processes are triggered and finally the cells adapt and resume proliferation.

Upon hyperosmotic shock, one of the major adaptive strategies of the cell is to synthesize and accumulate small organic solutes to maintain cell volume. These solutes are either amino acids and derivatives, polyols and sugars, methylamines, methylsulfonium compounds or urea. Except urea, they are often called ‘compatible osmolytes’, a term indicating lack of perturbing effects on cellular macromolecules and implying interchangeability. In contrast urea, the major organic osmolyte in marine elasmobranch fishes (ureosmotic animals), is a perturbing solute (Yancey, 2005). At the observed concentrations in these fishes and in mammalian kidneys (several hundred

millimolar) urea destabilizes many macromolecular structures and inhibits functions such as ligand binding. However, these animals have other osmolytes, mainly methylamines such as trimethylamine N-oxide (TMAO) and methylamines glycerophosphorylcholine (GPC), to counteract the deleterious effect of urea. Apparently, many organisms use mixtures of different osmolytes to counteract a hypertonic environment (Yancey, 2005).

1.3.1 The osmotic stress response in *Saccharomyces cerevisiae*

S. cerevisiae is the most commonly used system to study osmoadaptation mechanisms because of the industrial interest. Yeast cells respond to hyperosmotic conditions by a whole range of physiological changes. The cytoskeleton is reorganized, ion homeostasis is changed, metabolic processes are adapted, and the cell cycle is stopped (Hohmann, ; Posas et al., 2000; Rep et al., 2000).

A lot of work for the global gene expression analyses has been carried out in *S. cerevisiae*, which shed light on its comprehensive network of osmotic stress response. Some general conclusions can be drawn from these studies. i) The set of genes specific to hyperosmotic shock is rather small, but a large set of genes is differentially regulated under all environmental challenges, which is the general stress response. ii) Induction and repression of genes are important for *S. cerevisiae*, but the number of repressed genes is higher than that of induced genes. iii) The transcriptional response is transient and follows a distinct temporal pattern. iv) Cellular systems involved in the osmotic stress could be confirmed by the global gene expression analyses. However, expression changes may not necessarily reflect physiological changes. v) The number of functionally uncharacterized genes among those differentially regulated genes is remarkably high, about 50 to 60% (Hohmann, 2002).

An osmotic upshift causes a dramatic transcriptional response, affecting expression of about 10% of the yeast genes and the cells adapt to this adverse condition by synthesizing the compatible osmolyte glycerol (Causton et al., 2001; Gasch et al., 2000; Posas et al., 2000; Rep et al., 2000; Yale and Bohnert, 2001). In recent years it became clear that several parallel signaling pathways can get activated in response to changes in the environment and transmit the signal to the transcriptional machinery. One of the signaling pathways, the HOG (high osmolarity glycerol) signal transduction pathway, a MAPK (mitogen-activated protein kinase) pathway, plays a central role and

controls via different transcription factors the expression of more than 150 genes (Hohmann, 2002; O´Rourke et al., 2002) (Figure 4). It can be activated by either of two upstream pathways, the SHO1 and the SLN1 pathway, which converge on Pbs2, a MAPKK and scaffolding protein that brings together the other components of the MAPK cascade (de Nadal et al., 2002). SHO1 and SLN1 are putative yeast osmosensors and there is possibly a third one, Msb2 (Maeda et al., 1995; Maeda et al., 1994; O´Rourke and Herskowitz, 2002). Microarray analysis showed that Msb2 and SHO1 function in parallel and regulate identical gene sets in *hog*1 mutants (O´Rourke and Herskowitz, 2002). The downstream transcription factors Hot1 and Sko1 either induce or represse the expression of osmoresponse genes. Osmotic stress also leads to other cellular consequences, such as energy consumption, oxidative stress, amino acid starvation and cellular morphology changes. (Hohmann, 2002; Tsujimoto et al., 2000).

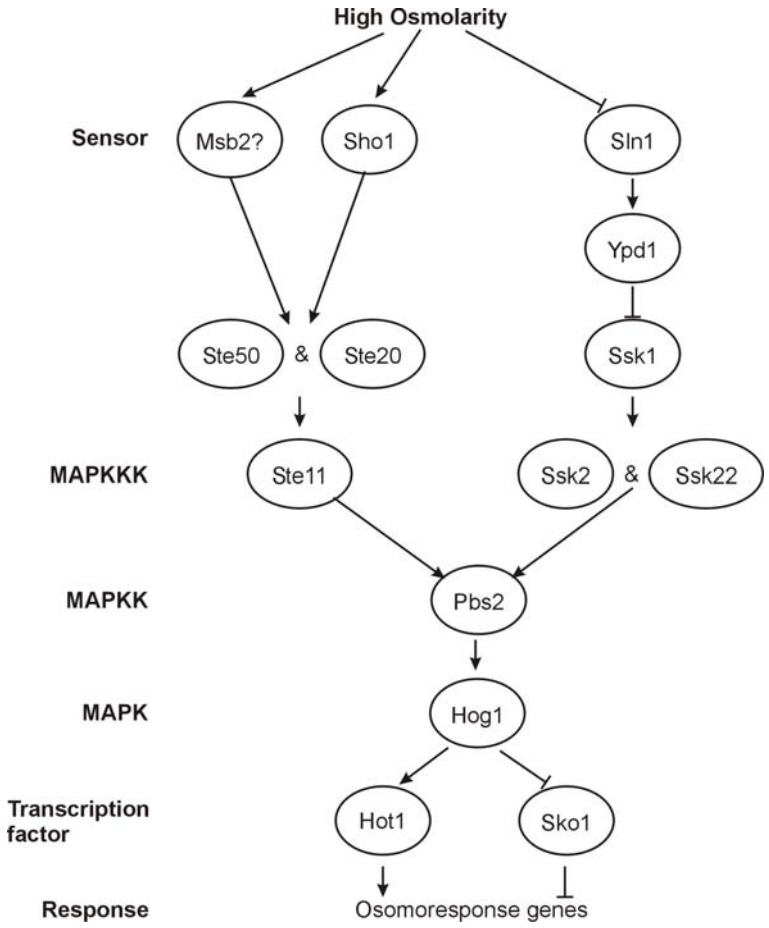

Figure 4. Osmoresponse HOG pathway in *S. cerevisiae*, adapted from (O´Rourke and Herskowitz, 2002) and modified.

1.3.2 The osmotic stress response in *Escherichia coli*

E. coli is another well-studied organism in the osmostress response realm. In an environment of elevated osmotic strength, *E. coli* accumulates K^+ and organic osmolytes, especially amino acids and their derivatives, from the environment (Epstein, 1986; Higgins et al., 1987; Strom et al., 1986). However, when growing in the absence of such organic osmolytes, it accumulates trehalose via endogenous biosynthesis and glutamate via suppression of glutamate catabolism (McLaggan et al., 1994). These organic or inorganic solutes have different importance for the osmoresponse. The accumulation of K^+ is more rapid and quantitatively most important in the initial phase. Since a high intracellular concentration of K^+ has negative effects on protein function and DNA-protein interactions, the accumulation of K^+ is an inadequate strategy for coping with prolonged high osmolarity. Therefore other systems, e.g. trehalose synthesis and uptake of glycine betaine, are induced and contribute to the restoration of turgor at later times of hyperosmotic stress (Poolman and Glaasker, 1998; Wood, 1999; Wood, 2006) (Figure 5).

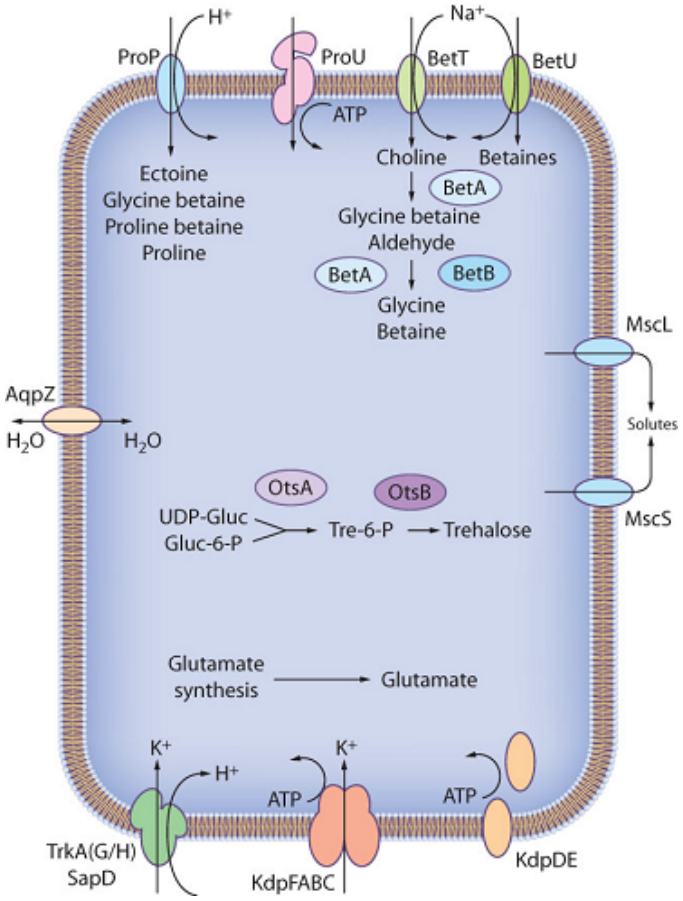

Figure 5. Osmoregulatory systems of *E. coli* (Wood, 2006). Initially Aquaporin (AqpZ) mediates transmembrane water flux, and K^+ transporters (TrkA(G/H)/SapD and KdpFABC) mediate K^+ accumulation. A two-component regulatory system (KdpDE), composed of an integral membrane sensor kinase and a cytoplasmic response regulator, controls the transcription of the K^+-channel (KdpFABC) in response to K^+ supply and osmotic stress. Suppression of glutamate catabolism leads to its accumulation as K^+ counterion. Membrane transporters (ProP, ProU, BetT, and BetU) mediate the import of the organic solutes proline, ectoine, choline and betaines. The enzymes BetA and BetB then convert choline and betaines to glycine betaine. In addition, the compatible osmolyte trehalose is synthesized from UDP-glucose and glucose-6-phosphate by the enzymes OtsA and OtsB.

1.3.3 The osmotic stress response in *D. discoideum*

In response to hypertonicity, *D. discoideum* cells shrink immediately, they round up and rearrange their cytoskeleton, which appears to play a key role in the initial protection of the organism from high osmolarity. Actin is tyrosine phosphorylated (Howard et al., 1993; Jungbluth et al., 1995) and myosin II is phosphorylated on three threonine residues in the tail region (Bosgraaf et al., 2002; Insall, 1996; Kuwayama et al., 1996; Roelofs and Van Haastert, 2002) (Figure 6).

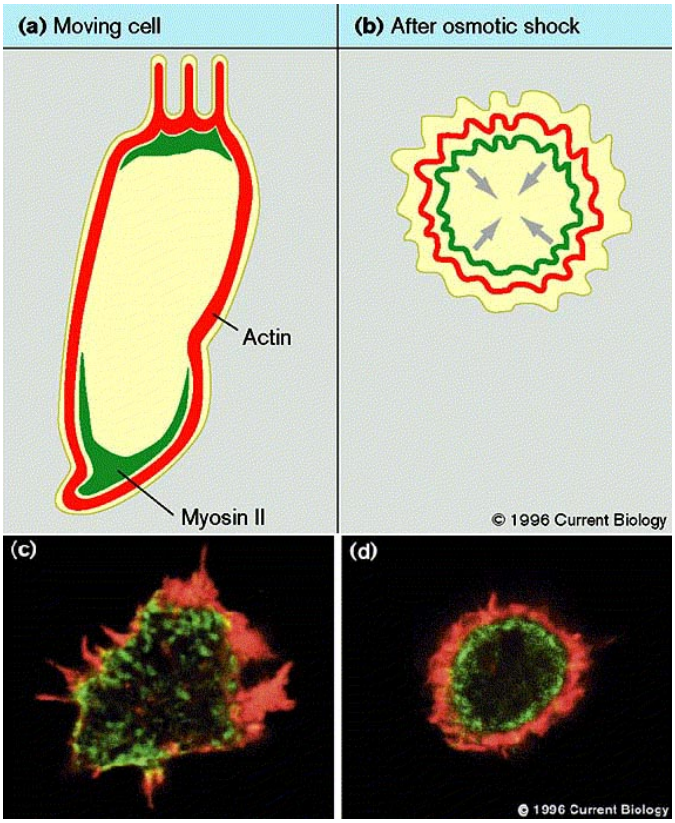

Figure 6 Cytoskeletal reorganization of *D. discoideum* in response to hyperosmotic shock (Insall, 1996). (a) In a moving wild-type cell, actin is found in the cortex and pseudopodia, and myosin II is at the rear and in the front. (b) After osmotic shock, the cell shrinks to half its normal size; the actin is in the cortex and the myosin II in a layer beneath the cortex. (c) Immunofluorescence of an unshocked cell, and (d) a cell shocked with 300 mM glucose for 10 min; actin is visualized in red and myosin II in green.

Neither the signal transduction chain nor the responsible protein kinase for actin phosphorylation is known, however, there is evidence that the phosphotyrosine phosphatase PTP1 is somehow involved in the dephosphorylation reaction (Howard et al., 1993) and that the small GTPase Rap1 is also involved in the regulation of actin phosphorylation in response to hyperosmotic shock (Kang et al., 2002). Myosin II phosphorylation appears to be triggered by the induction of soluble guanylate cyclase

(sGC) which leads to a rise in cGMP levels and the activation of myosin II heavy chain kinase possibly via the cGMP binding protein GbpC (Bosgraaf et al., 2002; Kuwayama et al., 1996; Roelofs and Van Haastert, 2002). Recent evidence suggests that Rap1 is involved in the cGMP response presumably by activating sGC (Kang et al., 2002). Phosphorylated myosin II disassembles from myosin filaments followed by cellular relocalization and reassembly. This apparently strengthens the cell cortex and is crucial for cell survival, as myosin II knock-out mutants and cells expressing mutant forms of myosin II, wherein the three threonine residues in the tail region were substituted by alanine, showed a dramatically reduced survival rate in high osmolarity (Kuwayama et al., 1996). Changes in the subcellular distribution of cell cortex proteins in response to sorbitol were also seen in two-dimensional gel electrophoresis with cytoskeletal and membrane fractions (Zischka et al., 1999). Furthermore, an increased sensitivity to hypertonicity was observed in double mutants of α-actinin and filamin, in hisactophilin mutants and in LimC, LimD and LimC/D mutants, supporting the importance of the actin cytoskeleton for the cellular resistance to an adverse osmotic environment (Khurana et al., 2002; Pintsch et al., 2001; Rivero et al., 1996).

A parallel pathway appears to be mediated by the hybrid histidine kinase DokA via a rise in intracellular cAMP levels. DokA minus cells showed a reduced viability on exposure to high osmolarity and artificial elevation of the intracellular cAMP concentration by 8-bromo-cAMP rescued this defect (Ott et al., 2000; Schuster et al., 1996). It is believed that activation of DokA by serine phosphorylation negatively regulates the RdeA:RegA two-component system which controls intracellular cAMP levels (Oehme and Schuster, 2001; Ott et al., 2000; Thomason and Kay, 2000). *In vitro* evidence suggests that DokA acts as a phosphatase for RdeA (Ott et al., 2000).

Another very important pathway involved in the hyperosmotic shock appears to be under the control of STATc. STAT (signal transducer and activator of transcription) proteins act as latent transcription factors and contain three highly conserved domains: a DNA binding site, an SH2 domain and a tyrosine phosphorylation site (Bromberg and Chen, 2001). A canonical STAT signalling pathway encompasses three major components: the cytokine receptor which is activated by extracellular stimuli, the Janus kinase (JAK) associates with the receptor and is activated, the STAT protein which is tyrosine-phosphorylated by JAK. STAT then dimerises and translocates to the nucleus

where it binds to the promoter region of target genes (Aaronson and Horvath, 2003) (Figure 7).

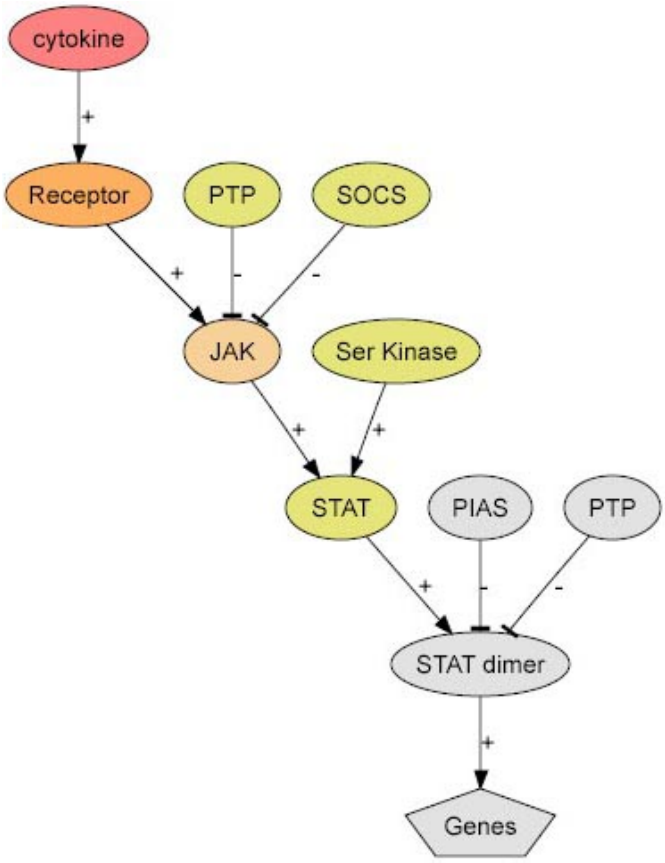

Figure 7 The JAK-STAT Pathway (Aaronson and Horvath, 2003). The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is capable of transmitting information from extracellular polypeptide signals (cytokine) through transmembrane receptors, directly from the cytoplasm to target gene promoters in the nucleus (genes). SOCS genes are induced by cytokine signals and their protein products feedback to JAK kinases to suppress cytokine signaling. STATs are also regulated by serine phosphorylation in their C-terminal transcriptional activation domains, which maximizes the transcriptional capacity of the STATs. Protein tyrosine phosphatase (PTP) can dephosphorylate and inhibit JAK and STAT. Another inhibitor of STAT is Protein inhibitor of activated STATs (PIAS). This canonical pathway presents the major themes common to most systems that use JAK-STAT signalling.

In vertebrates the JAK/STAT signalling pathway is activated by a large number of cytokines and growth factors. These signals induce proliferation or cell fate determination and are crucial to the proper growth and development of mammalian tissues (Hou et al., 2002). Interestingly, in mammalian cells, Jak1, Jak2, and Tyk2 (also a member of Janus kinase family) are tyrosine-phosphorylated and activate STAT1 and/or STAT3 in response to osmotic shock (Gatsios et al., 1998).

Introduction

Dictyostelium has four STAT members (STATa, b, c, d) in the genome, but only Dd-STATa and Dd-STATc have been studied in detail. Dd-STATa is activated by extracellular cAMP through the cAR1 (cAMP receptor 1) receptor during early development, binds to regulatory regions of target genes (for instance, the repressor element of *ecmB*), and thereby affects stalk cell differentiation (Kawata et al., 1997). Dd-STATc regulates the speed of early development and the timing of terminal differentiation. Developing *Dictyostelium* cells produce a chlorinated hexaphenone, DIF, which directs prestalk cell differentiation. In response to DIF STATc is activated by tyrosine phosphorylation, it dimerises, translocates to the nucleus and negatively regulates *ecmA* (a common marker used for prestalk cell differentiation) expression (Fukuzawa et al., 2001). Recent work showed that STATc, which is present in growing cells and throughout development, is also activated by osmotic stress (Araki et al., 2003). The link between STATc and the cAMP and cGMP signalling pathways is unclear. Although cGMP appears to be upstream of STATc, tyrosine phosphorylation of STATc was still observed in a *Dictyostelium* mutant wherein both known guanylate cyclases (GCA and sGC) were disrupted (Araki et al., 2003). In this mutant, guanylate cyclase activity falls below detectable levels. Furthermore, DokA and protein kinase A (PKA) do not act upstream of STATc and cGMP accumulates after hyperosmotic stress in the *dokA* mutant (Araki et al., 2003; Schuster et al., 1996).

As in other organisms, “omics” methods were also applied to study the osmostress response in *Dictyostelium*. However, the outcome is limited or even confusing. At the transcriptional level, only two genes (*rtoA* and *gapA*) were reported to be differentially regulated (Araki et al., 2003). However this result was based on a microarray that carried only 334 probes. At the translational level, hyperosmotic stress induced changes of the cytoskeleton and the crude membrane fraction but not of the whole-cell protein composition. It was also shown that the ubiquitination of cellular proteins was induced. In contrast to the general knowledge of hyperosmotic shock response in other organisms, it was claimed that *Dictyostelium* does not produce compatible osmolytes and does not undergo a regulatory volume increase (Zischka et al., 1999). To the contrary, Steck et al. demonstrated that the total intracellular amines increased with osmolarity, reaching values of 80 mM or more in media of >300 mOsM,

indicating the accumulation of intracellular free amino acids as compatible osmolytes (Steck et al., 1997).

1.4 The aim of the work

To date, the osmotic stress response has been intensively studied in many model organisms. The results yielded important information of the signaling pathways. However, only little information on the signal transduction pathways of *Dictyostelium* cells in response to this adverse condition was available. The comprehensive network of the regulators and the details of their actions, including the signals that activate them and the downstream targets they regulate, remained to be elucidated. Based on the evolutionary position of *Dictyostelium* we also reasoned that it could serve as a good model system to reveal additional signaling mechanisms that might be relevant to higher eukaryotes including human. It was therefore interesting to investigate the osmotic stress response in *D. discoideum* using the newly established DNA microarray platform in our lab. Using this tool, we attempted to address the following questions:

- What is the transcriptional response of *D. discoideum* cells to hypertonicity after a fixed time point and in a time course experiment?
- Which major biological processes are adapted by the cells?
- Can we infer metabolic changes based on the analysis of differentially regulated genes?
- Does the differential regulation of osmostress responsive genes give hints to the production of osmolytes?
- Which signalling pathways are involved in the osmostress response in *Dictyostelium*?

2. Material and Methods

Details regarding the following procedures are not included in this section:
Standard molecular biological techniques were carried out as described (Sambrook et al., 1989).
Instruments used were from the departmental facility.

2.1 Material

2.1.1 Lab material

Centrifuge tubes, 15 ml, 50 ml	Greiner
Coverslip (glass)	Roth
Coverslip (glass), Ø12 mm	Assistant
Gel drying frames	Novex
Hybridization tube	Hybaid
Microcentrifuge tube, 1.5 ml, 2.2 ml	Sarstedt
Micropipette, 1-20 µl, 10-200 µl, 100-1,000 µl	Gilson
Microcapillary, 100 µl	BLAUBRAND® intraMARK
Micropipette tips	Greiner
Nitrocellulose membrane, BA85	Schleicher and Schuell
Parafilm	American National Can™
Pasteur pipette, 145 mm, 230 mm	Volac
PCR softtubes, 0.2 ml	Biozym
Petri dish, 35 mm, 60 mm, 100 mm	Falcon
Petri dish, 90 mm	Greiner
Plastic cuvette	Eppendorf
Plastic pipettes (sterile)	Greiner
Poly-L-Lysine slide	Sigma
Microscope slide	Menzel
Syringes (sterile), 1 ml	Amefa, Omnifix
Sterile filter, 0.2 µm	Gelman Science
UltraGAPS microarray slide	Corning

Whatman 3MM filter paper	Whatman
X-ray film, X-omat AR-5, 18 x 24 mm, 535 x 43 mm	Kodak

2.1.2 Chemicals

1kb plus DNA ladder	Invitrogen
Agarose	Biozym
Boric acid	Merck
Bromophenol blue	Serva
BSA	Roth
Cyanin3 and Cyanin5	Amersham
DEPC	Sigma
DMSO	Merck
EDTA	Merck
Ethanol	Riedel-de Haen
Ethidium bromide	Sigma
Fish DNA	Roche
Formaldehyde	Sigma
Formaldehyde solution 37 %	Calbiochem
Formamide	Merck
Yeast extract	Oxoid
HEPES	Biomol
MOPS	Gerbu
Pepton	Oxoid
Phenol	Roth
Potassium dihydrogen phosphate	Merck
SDS	Serva
SeeBlue Pre-Stained Protein Standard	Invitrogen
Sodium acetate	Merck
Sodium azide	Merck
Sodium chloride	Fluka
Sodium bicarbonate	Merck
Sodium phosphate	Merck

Sodium hydroxide	Riedel-de Haen
TEMED	Roth
TRIS	Fluka, Riedel-de-Haen
Tween 20	Roth

2.1.3 Kits

FairPlay Microarray Labeling Kit	Stratagene
RNeasy Mini kit	Qiagen
RNeasy Midi kit	Qiagen
SpotReport-10 Array Validation System	Stratagene
Quantitect™ SYBR® green real time PCR kit	Qiagen
QIAprep Spin Miniprep kit	Qiagen
QIAGEN Plasmid Midi kit	Qiagen
QIAquick Gel Extraction kit	Qiagen
QIAquick PCR Purification kit	Qiagen

2.1.4 Radioactive chemicals

α-^{32}P-deoxyadenosine triphosphate, (10 mCi/ml)	Amersham

2.1.5 Enzymes, antibodies, substrates and antibiotics

2.1.5.1 Enzymes for molecular biology

StrataScript® Reverse Transcriptase	Stratagene
RNasin® Ribonuclease Inhibitor	Promega
Taq polymerase	Roche
Klenow fragment	Roche
Restriction endonucleases	Amersham New England Biolabs
T4 DNA Ligase	Promega

2.1.5.2 Antibodies

Mouse monoclonal anti-actin mAB Act-1-7	(Simpson et al., 1984)
Mouse monoclonal anti-Phosphotyrosine mAB,	

clone 4G10®	Millipore
Goat anti-mouse IgG, peroxidase conjugated	Sigma
Goat anti-mouse IgG, Cy5 conjugated	Sigma

2.1.5.3 Antibiotics

Ampicillin	Gruenenthal
Blasticidin S	ICN Biomedicals
Dihydrostreptomycinsulphate	Sigma

2.1.6 Oligonucleotides

Oligonucleotide primers used for PCR were designed on the basis of sequence and purchased from Metabion (Martinsried).

Table 2 Oligonucleotides

Name	Sequence
SLC675-F	5'- TACGATTGTGCCTACCAAATCG -3'
SLC675-R	5'- GTGTTGTATGAACCAGCTGGAG -3'
SSE751-F	5'- ATAATGTCAAATTGGGAACACG -3'
SSE751-R	5'- AGTTCATGAAAAGACCAGATGG -3'
VSA281-F	5'- CTCGTTGGTCTCTACAAGGAAG -3'
VSA281-R	5'- CACCTTGGACTAAGAAGGTAGC -3'
VSG596-F	5'- GGATCAGCTAAATCCATTTGGG -3'
VSG596-R	5'- AAATTGGACATGCATCTTTTGC -3'
SSL284-F	5'- ATGGTTCATCACAATCTGGATC -3'
SSL284-R	5'- ATTTAATGGTGACGAAGACCTC -3'
PkyA_5' forward	5'- GAAGGTATGGATCCGATATTGGC -3'
PkyA_5' reverse	5'- CGCCTGCAGGCAGGTGGTAAATTTGTAATTG-3'
PkyA_3' forward	5'- CGCAAGCTTCTTTAGGTATGGAACATCTTC-3'
PkyA_3' reverse (3' CTRL_RI)	5'- CGCGTCGACCTAACTATCAACCTCTTCATC-3'
PkyA_3' CTRL_RII	5'- GTATTTCCAATCAAGACAACACATAAAAG-3'
PkyA_3' CTRL_F	5'- GTCGCTACTTCTACTAATTCTAGATCTTGTTG-3'
DDB0231199_5' forward (5' CTRL_FI)	5'- CGCGGATCCCCAATAGGTTCTATCGCATC-3'
DDB0231199_5' reverse	5'- CGCCTGCAGCCAATCCTCCATCATATCTTTT-3'
DDB0231199_3' forward	5'- CGCATCGATGCTTGTATTGCACCTCGTTC-3'
DDB0231199_3' reverse	5'- CGCGTCGACGGACGTTTCTTTGGAGAATT-3'
DDB0231199_5' CTRL_FII	5'- CAGAAACAATAGCATCATCAGGG-3'
DDB0231199_5'_R	5'- CTCATTCCACTCAAATATACCCG-3'

2.1.7 Media and Buffers

All media and buffers were prepared with deionized water, filtered through an ion-exchange unit (Membra Pure). The media and buffers were sterilized by autoclaving at 120°C and antibiotics were added to the media after cooling to approx. 50°C. For making agar plates, a semi-automatic plate-pouring machine (Technomat) was used.

AX2 medium, pH 6.7 (Claviez et al., 1982)

7.15 g yeast extract, 14.3 g peptone (proteose), 18.0 g maltose, 0.486 g KH_2PO_4, 0.616 g $Na_2HPO_4 \cdot 2H_2O$, add H_2O to make 1 liter

Phosphate agar plates, pH 6.0

9 g agar, add Soerensen phosphate buffer, pH 6.0 to make 1 liter

Salt solution (John Tyler Bonner, 1947)

10 mM NaCl, 10 mM KCl, 2.7 mM $CaCl_2$

SM agar plates, pH 6.5 (M. Sussman, 1951)

9 g agar, 10 g peptone, 10 g glucose, 1 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$, 2.2 g KH_2PO_4, 1 g K_2HPO_4, add H_2O to make 1 liter

Soerensen phosphate buffer, pH 6.0 (Malchow et al., 1972)

2 mM Na_2HPO_4, 14.6 mM KH_2PO_4

10× MOPS, pH 7.0 / pH 8.0

41.9 g MOPS, 7 ml 3 M sodium acetate, 20 ml 0.5 M EDTA, add H_2O to make 1 liter

10× NCP buffer, pH 8.0

12.1 g Tris/HCl, pH 8.0, 87.0 g NaCl, 5.0 ml Tween 20, 2.0 g sodium azid, add H_2O to make 1 liter

NP-40 buffer

0.05M HEPES, 0.05 M MgAc, 10% sucrose, 2% Nonidet P40 (NP40)

1× PBS, pH 7.4

8.0 g NaCl, 0.2 g KH_2PO4, 1.15 g Na_2HPO4, 0.2 g KCl dissolved in 900 ml deionized H_2O, adjust to pH 7.4, add H_2O to make 1 liter, autoclave

20× SSC, pH 7.0

3 M NaCl, 0.3 M sodium citrate

TE buffer, pH 8.0

10 mM Tris/HCl, pH 8.0, 1 mM EDTA

PBG, pH 7.4

0.5% bovine serum albumin, 0.1% fish gelatin in 1× PBS, pH 7.4

PBS Glycine

500 ml 1× PBS, 3.75 g glycine, filter sterilized and stored at –20°C

Electroporation buffer

10 mM K-PO_4, pH 6.1, 50 mM glucose, filter sterilized

Healing solution

100 mM $CaCl_2$, 100 mM $MgCl_2$

2.1.8 Biological materials

Klebsiella aerogenes (Williams and Newell, 1976)

E.coli DH5α (Hanahan, 1983)

pLPBLP plasmid (http://dictybase.org/Vector_sequences_web/pLPBLP.txt)

D. discoideum strain AX2-214, an axenically-growing derivative of the NC-4 isolate (Raper, 1935)

SrfA null mutant (Escalante and Sastre, 1998)

STATc null mutant (Fukuzawa et al., 2001)

STAT-RIC strain (J. Williams, personal communication)

2.1.9 Computer program

ScanArray Express 3.0 (PerkinElmer Life Sciences, Wellesley, USA)

R 1.6.2 BioConductor, http://www.bioconductor.org/

Significance Analysis of Microarrays (SAM) 1.21 (Tusher et al., 2001)

GeneSpring 7.2 Agilent Technologies, http://www.chem.agilent.com

Gene Ontology Analysis Tool (GOAT) (Xu and Shaulsky, 2005)

Array tools (http://www.uni-koeln.de/med-fak/biochemie/transcriptomics/tools.e.shtml)

Compare (http://www.uni-koeln.de/med-fak/biochemie/transcriptomics/tools.e.shtml)

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Growth of *D. discoideum*

The procedure was adopted from Claviez et al. (Claviez et al., 1982). *D. discoideum* AX2 and the derived transformants were grown in liquid AX2 medium containing dihydrostreptomycinsulfate (40 µg/ml) and other appropriate selective antibiotics (depending on the mutant) at 21°C either in a shaking suspension in Erlenmeyer flasks with shaking at 160 rpm or the cells were grown on petri dishes. For cell biological work, cultures were harvested at a density of 3-4 x 10^6 cells/ml.

2.2.1.2 Hyperosmotic shock of *D. discoideum*

Dictyostelium cells were grown to a density of 3-4 x 10^6 cells/ml in Erlenmeyer flasks. 2 M sorbitol was added to the culture for a final concentration of 200 mM sorbitol. Samples were collected after treatment for 60 min or, for the time course experiments, samples were collected at 0, 15, 30, 45, 60, 90, and 120 min. As control, soerensen phosphate buffer was added to the culture.

2.2.1.3 Determination of cell survival

Dictyostelium cells were treated with 200 mM sorbitol as described above, serial dilutions were performed and approximately 100 *Dictyostelium* cells were plated onto SM agar plates overlaid with *K. aerogenes*. *Dictyostelium* plaques were counted after 2-3 days of incubation at 21°C.

2.2.1.4 Determination of cell volume

Dictyostelium cells were treated with 0, 50, 100, 200 or 400 mM sorbitol for 5 minutes. Then cells were transferred to a 100 µl microcapillary tube (BLAUBRAND® intraMARK) and centrifuged at 500×g for 1 minute. The height of the cell pellet in the microcapillary was taken as a measure for cell volume.

2.2.1.5 Indirect immunofluorescence microscopy study of *Dictyostelium*

2.2.1.5.1 Preparation of *Dictyostelium*

Dictyostelium cells were harvested at a density of 3-4 x 10^6 cells/ml, washed twice with soerensen phosphate buffer and finally resuspended in the same buffer at 1 x 10^6 cells/ml. Cells were starved for 4 hours in soerensen phosphate buffer. 300 µl of the cell suspension were then pipetted onto Ø12 mm coverslips resting on a petri dish. Cells were allowed to attach to the coverslip for 30 minutes, treated with sorbitol (0, 50, 100, 200 and 400 mM) for 5 minutes and then fixed immediately as described below.

2.2.1.5.2 Methanol fixation

Dictyostelium cells on the coverslip were incubated with pre-chilled (-20°C) methanol in the petri dish for 10 minutes. The coverslip was then washed three times with 500 µl PBS/glycine for 5 minutes to block free reactive groups, followed by two washings with 500 µl PBG for 15 minutes. *Dictyostelium* cells were immunostained as described below.

2.2.1.5.3 Immunostaining

The coverslip was incubated with 300 µl of the primary antibody (diluted in PBG) for 1-2 h in a humid-box at room temperature. After incubation, the excess antibody was removed by washing the coverslip five times with PBG for 5 minutes each. The coverslip was then incubated for 1 hour with 300 µl of the appropriate secondary antibody (diluted in PBG) followed by two washes with PBG for 5 minutes and then three washes with PBS for 5 minutes.

2.2.1.5.4 Mounting

After immunostaining, the cover slip was swirled once in deionized water and the extra water was blotted off on a soft tissue paper and embedded carefully with gelvatol. The mounted slide was then stored in the dark at 4°C for overnight. Thereafter, the mounted slide was observed under a confocal laser scanning microscope (Leica DM/IRBE).

2.2.1.6 Transformation of *Dictyostelium* cells by electroporation

The electroporation method for transformation of *Dictyostelium* cells described by de Hostos et al. (de Hostos et al., 1993) was followed with little modifications. *Dictyostelium* cells were grown axenically in suspension culture to a density of 2-3 x 10^6 cells/ml. The cell suspension was incubated on ice for 15 min and centrifuged at 500 x g for 2 min at 4°C to collect the cells. The cells were then washed with an equal volume of ice-cold Soerensen phosphate buffer and afterwards with an equal volume of ice-cold electroporation buffer. After washing, the cells were resuspended in electroporation buffer at a density of 1 x 10^7 cells/ml.

For electroporation, 35 µg of linearized plasmid DNA was added to 700 µl of the cell suspension and the cell-DNA mixture was transferred to a pre-chilled electroporation cuvette (4 mm electrode gap, Bio-Rad). Electroporation was performed with an electroporation unit (Gene Pulser, Bio-Rad) with the following settings (square wave, V=1.0 kV, 10 µF, 1.0 ms pulse length, 2 pulses, 5 sec pulse interval).

After electroporation, the cells were immediately spread onto a 100-mm petri dish and were allowed to sit for 15 min at 21°C. Thereafter, 15 µl healing-solution was added dropwise onto the cells and the petri dish was incubated at 21°C on a shaking platform at 40 rpm for 15 min. 12 ml of AX2 medium was added into the petri dish and the cells were allowed to recover overnight. The next day, the medium was replaced by the selection medium containing 10 µg/mL Blasticidin S. To select stable transformants, the selection medium was replaced every 24-48 hr until colonies of 1 mm diameter were clearly visible. At this time the control plate (containing cells electroporated without any DNA) was clear of live cells.

Viable colonies were serially diluted and plated onto SM agar plates overlaid with *K. aerogenes* to obtain single clones.

2.2.2 Biochemical methods

2.2.2.1 Preparation of total protein from *Dictyostelium*

4 x 10^6 *Dictyostelium* cells either treated or untreated with sorbitol were washed twice in Soerensen phosphate buffer. Total protein was prepared by lysing the pellet of cells in 200 µl 1 x SDS sample buffer and boiling 3 minutes at 95 °C. Equal amounts of protein (equivalent to 2 x 10^5 cells/lane) were loaded onto discontinuous SDS-polyacrylamide gels.

2.2.2.2 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the discontinuous buffer system (Laemmli et al., 1970).

2.2.2.3 Western blotting

The proteins resolved by SDS-PAGE were electrophoretically transferred from the gel to the nitrocellulose membrane at 15 V for 45 minutes (Kyhse-Andersen, 1984). The membrane was blocked with 1% milk in 1 x NCP and probed with different dilutions of the respective primary antibodies and POD-conjugated secondary antibodies. Antigens were detected by ECL (enhanced chemi-luminescence, Amersham).

2.2.3 Molecular biological methods

2.2.3.1 Isolation of genomic DNA from *Dictyostelium* cells

2.2.3.1.1 For PCR purpose

Genomic DNA was extracted with the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturers instruction.

2.2.3.1.2 For Southern blotting purpose

10^8 *Dictyostelium* cells were harvested and wash twice with cold water. The cell pellet (~2-4 g) was resuspended in 10 ml ice-cold NP-40 buffer and vortexed vigorously for 30 seconds. Nuclei were sedimented by 10 minutes centrifugation at 8,000 x g. The nuclei were resuspended in 3.8 ml TE buffer, and lyse by 0.2 ml 10% SDS. Protein was digested by incubation with proteinase K to 200 µg/ml at 37 °C for overnight. DNA was purified by phenol/chloroform/isoamyl alcohol (25:24:1) extraction, then precipitated by the addition of ice-cold ethanol and dissolved in an appropriate volume of TE buffer.

2.2.3.2 Isolation of total RNA from *Dictyostelium* cells

Dictyostelium cells, treated with sorbitol or untreated, were harvested and washed twice with Soerensen buffer. Total RNA was extracted with the Qiagen RNeasy® Midi/Mini Kit according to the "Protocol for Isolation of Cytoplasmic RNA from Animal Cells". After isolation, the RNA concentration and RNA quality were determined by measuring the OD260 and OD280, and further confirmed by denaturing agarose gel electrophoresis essentially as described (Lehrach et al., 1977).

2.2.3.3 Northern blotting

Total RNA (10 µg) was separated on a 1.2% denaturing agarose gel, transferred to nitrocellulose and hybridized as described (Noegel et al., 1985).

2.2.3.4 Southern blotting

Genomic DNA (12 µg) was separated on a 0.8% agarose gel, transferred to nitrocellulose and hybridized as described in Sambrook et al (Sambrook et al., 1989).

2.2.3.5 Generation of cDNA

cDNA was prepared by reverse transcription of 5 µg RNA with oligo dT (18) using StrataScript® reverse transcriptase (Stratagene) according to the protocol of the manufacturer.

2.2.3.6 Real time PCR

Real time PCR was carried out with the Opticon II instrument (MJ Research) using the Quantitect™ SYBR® green PCR kit (Qiagen, Hilden, Germany). Gene-specific primers of 22–26 bases directed against the 3' region of the corresponding genes were selected with the program GeneFisher (http://bibiserv.techfak.uni-bielefeld.de/genefisher/) for product sizes of 200–400 bases. PCR was performed for 60 cycles at 94°C for 60 s (denaturation), 58°C for 45 s (annealing) and 72°C for 45 s (elongation). As a quantification standard defined concentrations of the annexin VII gene (Doring et al., 1995) cloned into the pT7-7 vector (Tabor, 1990) were used for amplification.

2.2.3.7 Construction of the knockout vector

The gene of interest was disrupted by targeted homologous recombination. Suitable 5' and 3' fragments of the gene were amplified with primers incorporated with appropriate restriction sites, and the products cloned into pLPBLP vector flanking the floxed Bsr cassette (Kimmel and Faix, 2006). The vector, composed of Bsr cassette, 5' and 3' sequences of the gene of interest, was linearized by digestion with appropriate restriction enzyme, purified and then used for electroporation.

2.2.4 Microarray methods

We employed cDNA microarrays that carry a non-redundant set of 5,423 EST clones that were selected as part of the Dictyostelium cDNA project (Urushihara et al., 2004). In addition, appropriate positive and negative controls as well as partial sequences of 450 selected genes were present on the array (Farbrother et al., 2006). All probes were spotted in duplicate. A complete description of the microarray dataset is available at the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo; accession number GPL1972). Microarray production has been performed essentially as described (Farbrother et al., 2006).

2.2.4.1 Target preparation

Target preparation was performed by reverse transcribing 20 µg of total RNA per reaction in the presence of aminoallyl dUTP with the FairPlay™ Microarray Labeling Kit (Stratagene, La Jolla, USA), and the cDNA was labeled with activated Cy™3 and Cy™5 fluorescent dyes (Amersham Biosciences, Uppsala, Sweden). Dye swaps were

performed for the labelling of the cDNA from each independent isolation. Cy™3 and Cy™5 labelled targets were mixed, ethanol precipitated and dissolved in 65 µl of hybridization buffer (Noegel et al., 1985) with 500 µg/ml Fish sperm DNA (Roche, Mannheim, Germany) and 2 µM oligo dA 18-mer.

2.2.4.2 Microarray hybridization and scanning

The hybridization mix was heated to 80 °C for 10 min, applied to the microarray under a cover-slip and incubated in a hybridization chamber (Corning, New York, USA) for 15 hours at 37 °C. Post-hybridization washes were performed twice with 2 x SSC, 0.1 %SDS and once with 0.1 x SSC, 0.1 %SDS for 5 min each, five times with 0.1 x SSC and once with 0.01 x SSC for 5 sec each and dried by centrifugation at 235 x g for 5 min. Signal detection was performed with the ScanArray® 4000XL confocal laser scanner (PerkinElmer Life Sciences, Wellesley, USA). Two image pairs were produced per microarray slide, one with high laser intensity so that signals for most probes and also some saturated signals were obtained and a second one with lower laser intensity so that none of the signals was saturated. This way the dynamic range of the measurement was expanded. Images for Cy™3 and Cy™5 were obtained, spots were detected and quantified with ScanArray® Express v3.0 (PerkinElmer Life Sciences), then manually inspected and if necessary corrected.

2.2.4.3 Data analysis

Array tools (http://www.uni-koeln.de/med-fak/biochemie/transcriptomics/tools-array.e.shtml) was used to handle the import and export of microarray data to different analysis programs. Upon import of two data files of the same microarray scanned with different laser powers the saturated spots of the high laser power scan were replaced by non-saturated spots from the low laser power scan. In addition the import also performed data filtering by flagging SpotReport® controls, negative controls, empty spots, spots where only spotting solution was printed and spots whose intensities were below or equal to zero as 'Bad'.

Fluorescence ratios were normalized by LOWESS-normalization using R 1.6.2 (BioConductor, http://www.bioconductor.org/). Differentially expressed genes were identified with the program Significance Analysis of Microarrays (SAM) (Tusher et al., 2001). Differentially regulated genes that were common between the different experiments were detected with the program “compare” (http://www.uni-koeln.de/med-

fak/biochemie/transcriptomics/tools.e.shtml). Cluster analysis was performed with GeneSpring 7.2 (Agilent Technologies, http://www.chem.agilent.com).

GO term enrichment was analyzed with GOAT (Xu and Shaulsky, 2005). A complete list of all *Dictyostelium* proteins with GO annotations is available from the GO website (http://www.geneontology.org/GO.current.annotations.shtml). To identify enriched GO terms we selected those genes of the array (reference list) and of the identified clusters (gene lists), respectively, whose gene products have GO annotations. Given a gene and a reference list, the GOAT program calculates the enrichment and statistical significance of every GO term by comparing the observed number of genes in a specific category with the number of genes that might appear in the same category if a selection performed from the same reference list were completely random.

3 Results

3.1 High osmolarity triggers a variety of responses in *Dictyostelium* cells

The cellular response of *Dictyostelium* cells to treatment with sorbitol was analyzed with different methods.

3.1.1 Tyrosine phosphorylation of actin

It has been shown previously that hyperosmotic shock triggers actin tyrosine phosphorylation (Howard et al., 1993). We confirmed this biochemical response in a 2-hour time course of sorbitol treatment. Strong signals were observed at 15 and 30 minutes of treatment and a stepwise decrease during the remaining 90 minutes (Figure 8).

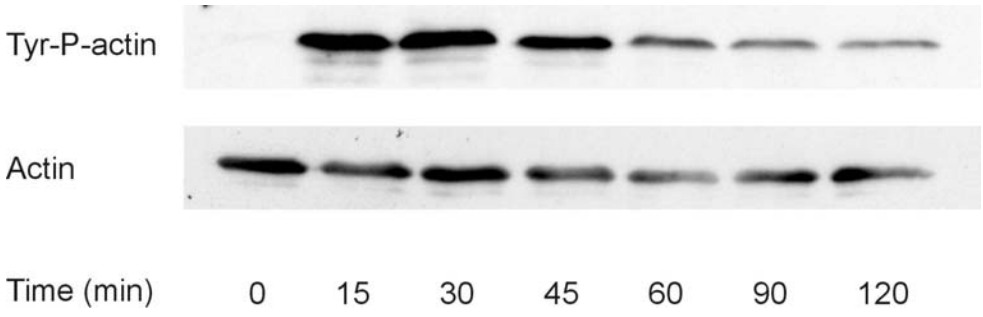

Figure 8. Tyrosine phosphorylation of actin. AX2 cells were shaken at a density of 3-4 x 10^6 in AX2 medium in the absence or presence of 200 mM sorbitol. At the indicated times, 1 ml aliquots were harvested, centrifuged and the pellet lysed in 1 x SDS sample buffer. Cell lysates corresponding to 2 x 10^5 cells were separated by SDS-PAGE, blotted onto nitrocellulose and probed with either a phosphotyrosine-specific monoclonal antibody or an actin-specific monoclonal antibody.

3.1.2 Redistribution of the F-actin cytoskeleton in response to hyperosmotic shock

Immunofluorescence studies of starved *Dictyostelium* cells with a monoclonal antibody directed against actin revealed the redistribution of the F-actin cytoskeleton in response to hyperosmotic conditions (Insall, 1996; Kuwayama et al., 1996). Our experiments confirmed this result. While untreated cells showed a polarized actin distribution, F-actin was redistributed in treated cells and appeared to form a continuous cortex at 400 mM sorbitol. The cortex is thought to play a pivotal role in protecting the

cell against the hyperosmotic environment. The analysis also showed that upon treatment the cells changed their shape and also decreased in size (Figure 9).

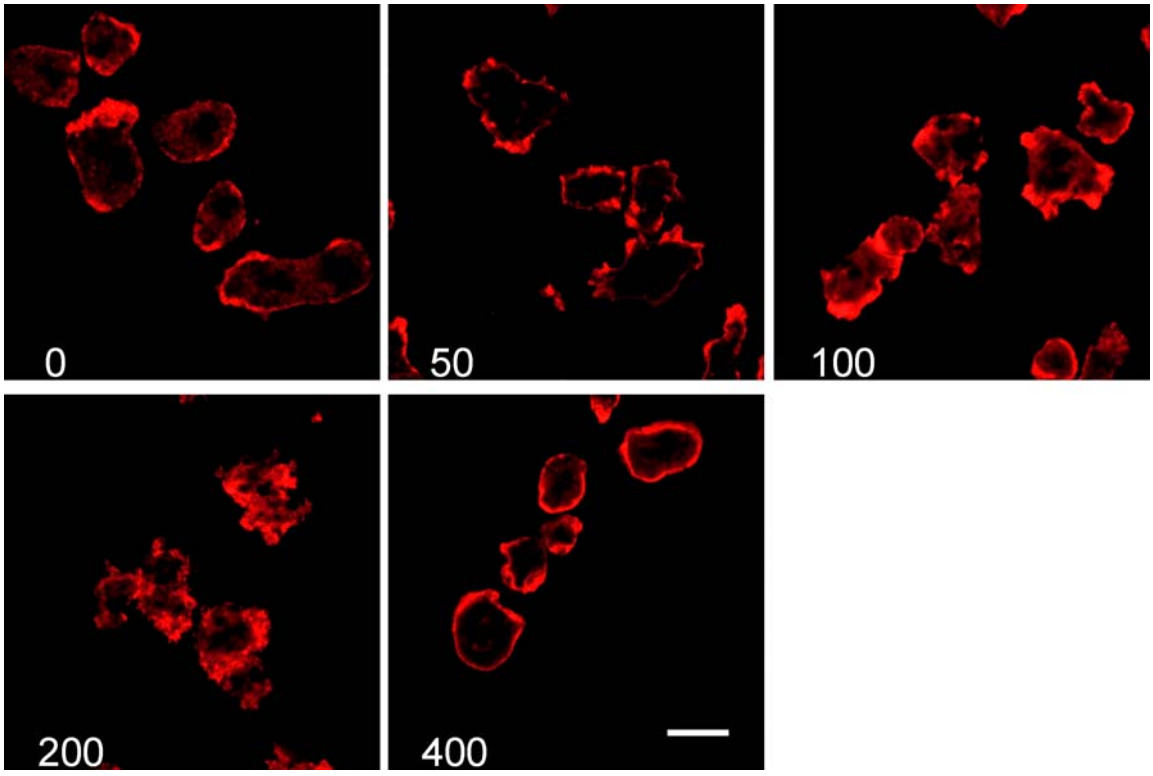

Figure 9. Redistribution of the F-actin cytoskeleton in response to hyperosmotic shock. After starvation for 4 hours, untreated cells and cells treated for 5 min with 50, 100, 200 and 400 mM sorbitol were fixed with ice cold methanol, and then stained with a monoclonal antibody specific for actin, followed by the incubation with anti-mouse IgG antibody conjugated with Cy5. Size bar is 10 µm.

3.1.3 Decrease of cell volume

We quantified the decrease in cell volume and found that it was strictly dependent on sorbitol concentration. At 50 mM sorbitol the cell volume decreased to 73 % and at 400 mM to 48 % of untreated cells. Extracellular concentrations of 100 and 200 mM sorbitol resulted in intermediate values (Figure 10). The decrease in cell size is due to loss of water resulting in a fast increase of the intracellular osmolarity of the treated cells until their osmolarity matches the surrounding medium (Kwon and Handler, 1995). In addition, we found that the *Dictyostelium* cells were able to adapt to 50 and 100 mM sorbitol and resumed the original cell size after about one hour of treatment (data not shown).

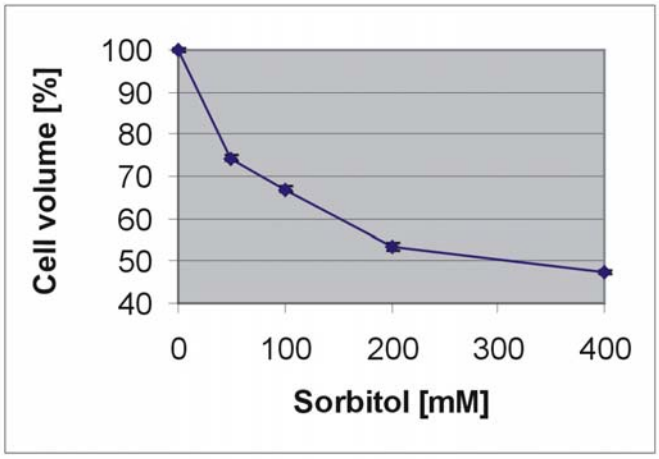

Figure 10. Decrease of cell volume in response to hyperosmotic condition. *Dictyostelium* cells were treated with indicated sorbitol concentrations for 5 minutes. Cell volume was measured with a microcapillary as described in material and methods [see 2.2.1.4]. Values represent the mean of three independent experiments ± standard deviation (SD).

3.1.4 Decrease of cell viability

Next we checked cell survival by treatment of the cells with 200 mM sorbitol for different times. After 15 and 30 minutes about 10 % and after 45 minutes about 25 % of the cells had died and the latter value remained constant throughout the time course (Figure 11).

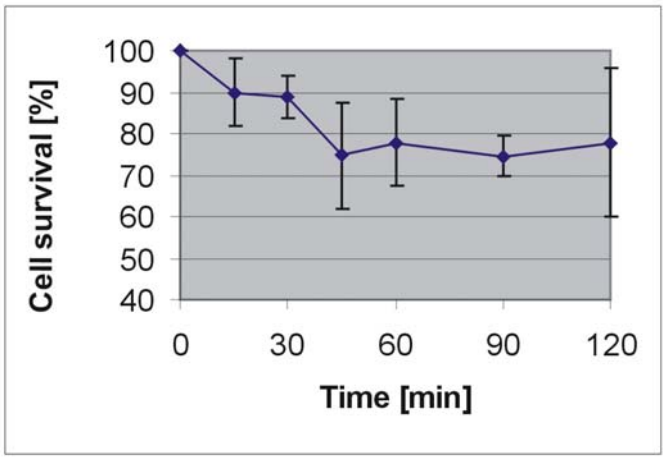

Figure 11. Cell survival in response to hyperosmotic shock. Cell survival was measured by plating out treated or untreated cells on *K. aerogenes* lawns and counting the plaques after 2 days of incubation at 21°C. Values represent the mean of three independent experiments ± SD.

These results exemplified the complex response of *Dictyostelium* cells to hyperosmotic conditions. To better understand this response we treated the cells with 200 mM sorbitol and analyzed their global transcriptional response by using DNA microarrays.

3.2 Hyperosmotic shock of *Dictyostelium* cells results in dramatic transcriptional changes

3.2.1 Treatment of *Dictyostelium* cells with 200 mM sorbitol for 1 hour leads to dramatic transcriptional changes

We employed cDNA microarrays to first analyze the transcriptional changes one hour after treatment with 200 mM sorbitol. In total 16 slides from 8 independent biological samples were hybridized and scanned. Figure 12 shows a section of one slide as an example.

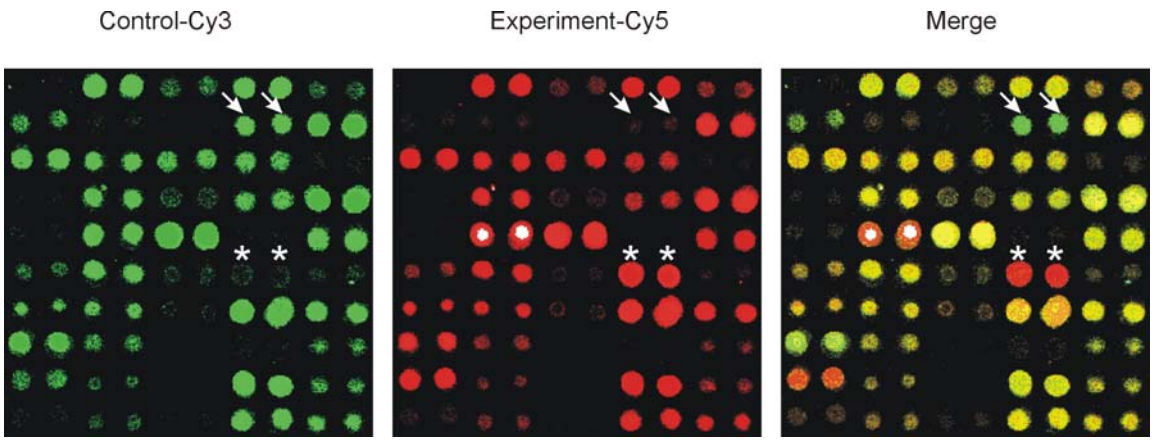

Figure 12. Section of the *Dictyostelium* cDNA microarray after hybridization and scanning. cDNA from control cells was labelled with Cy3 and experiment cells with Cy5. All probes were printed in duplicate. The arrows indicate a down-regulated gene (SSH169) and the stars indicate an up-regulated gene (SSG865).

After quantitation of the spot signals, the raw data were normalized by LOWESS-normalization using R 1.6.2 (BioConductor, http://www.bioconductor.org/) as described in material and methods [see 2.2.4.3]. The intensity of the spots in the two channels depends on the fluorescent dye coupling efficiency, the laser power for scanning and

other variables during experiment processing, which could lead to colour bias. In order to exclude the colour bias, the data have to be normalized. We used the global normalization method, which is based on the hypothesis that most genes do not change their expression during treatment. In this process the median M value [M= $Log2\frac{Intensity(\exp eriment)}{Intensity(control)}$] is adjusted to 0, and the M value of every spot is then normalized to this adjustment. Figure 13 shows the colour bias before normalization and after normalization. Some slides display an obvious colour bias before normalization, e.g. slides 1, 3 and 8, and the normalization procedure could correct this bias (Figure 13).

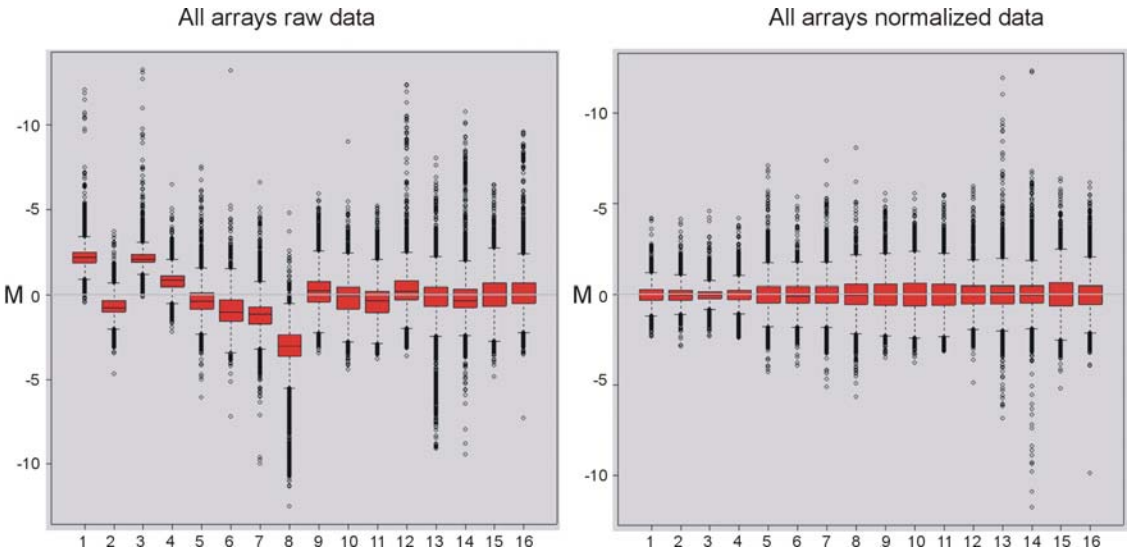

Figure 13. **Normalization of the microarray data.** The value distribution of 16 microarrays before (A) and after normalization (B) is represented. The red box covers 50% of the data points of one microarray and the line in the box indicates the median value of the distribution. The two lines above and below the red box limit the distribution and points beyond these lines are outliers. During the normalization the distributions of the spots are shifted based on a M-value of zero and the height of the distributions is adapted.

The normalized data were imported to SAM, which not only identifies the differentially regulated genes, but also predicts the number of false positives [see 2.2.4.3 Data analysis]. Without additional threshold SAM reported 2007 genes as differentially expressed, of which 873 were up-regulated and 1134 down-regulated (Figure 14).

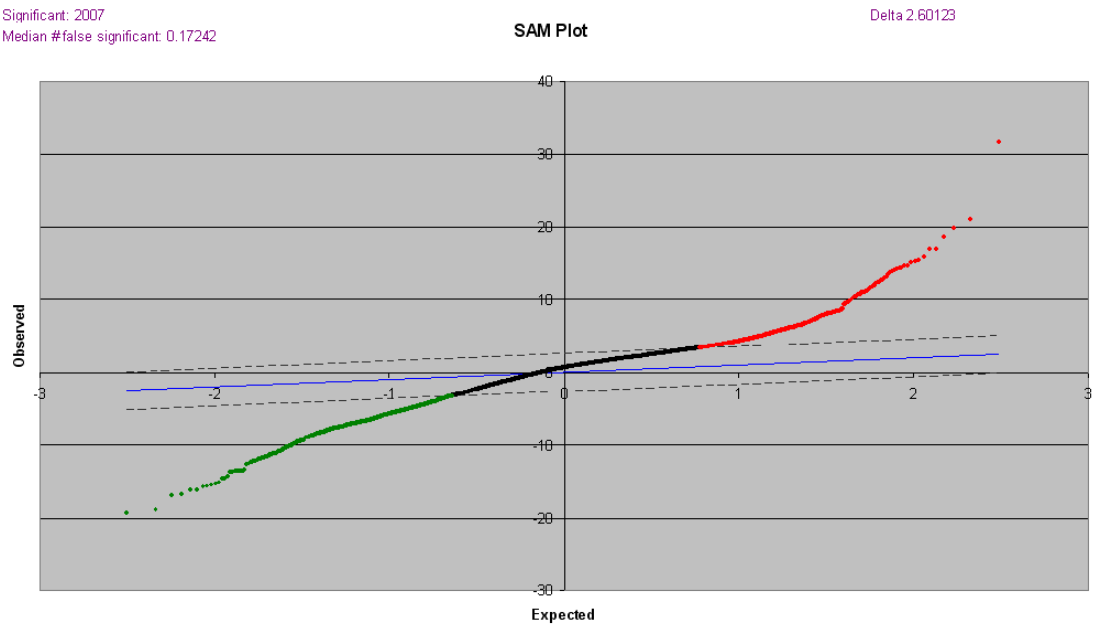

Figure 14. SAM diagram. The SAM t-statistic value of each gene (observed) is plotted versus the expected order statistics value (expected). A positive score shows up-regulated genes (red dots) and a negative score down-regulated genes (green dots). Delta is the threshold value for significant changes and is displayed as the intersection between the dashed lines. The number of significant genes and the percentage of false significant genes are both indicated (upper side, left).

Since many of those genes were only slightly up- or down-regulated and the biological significance of small changes in the expression pattern is not clear, we set in addition a threshold of 1.5 fold change. Under these conditions still 1188 genes were found to be differentially expressed, among which 441 were up-regulated and 747 were down-regulated. Significantly more genes were down-regulated than up-regulated which suggests that *Dictyostelium* down-regulates many cellular processes in order to survive hyperosmotic conditions. The threshold of 1.5 fold was used in the analysis of all further experiments.

3.2.2 Validation of microarray results with real time PCR and Northern blot

We randomly selected five of these genes to validate the microarray results by real time PCR. The differential expression was confirmed for all five genes (Figure 15).

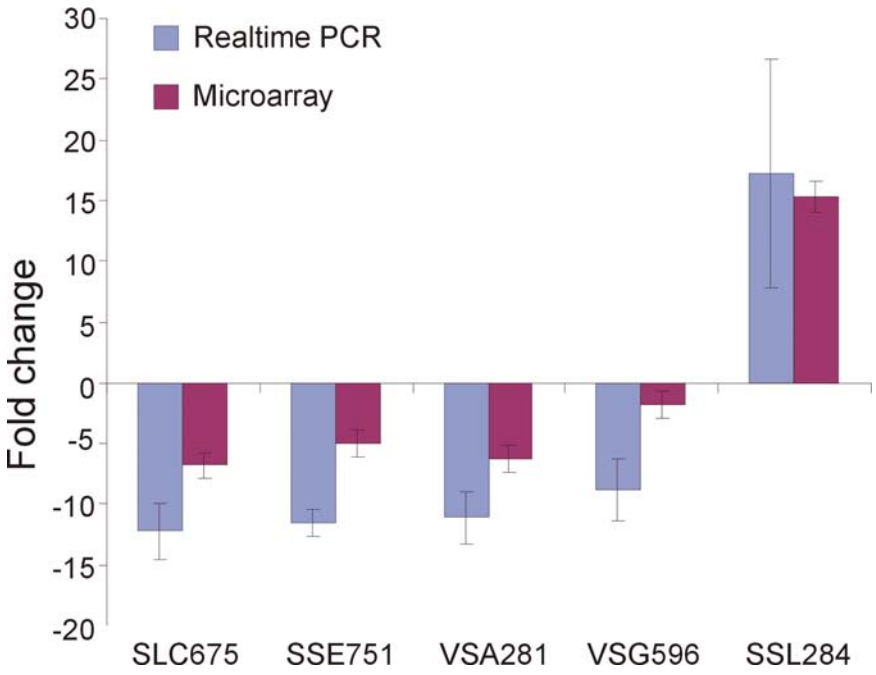

Figure 15. Five ESTs were analyzed by real time PCR and compared with the microarray results. The data are expressed as means of fold change ± SD of three independent experiments. The corresponding DDB IDs from left to right as follows: DDB0188166, DDB0235172, DDB0188166, DDB0190245, DDB0185120.

Furthermore, the expression of gene *sigJ* (DDB0191111) was confirmed by northern blot (Figure 16).

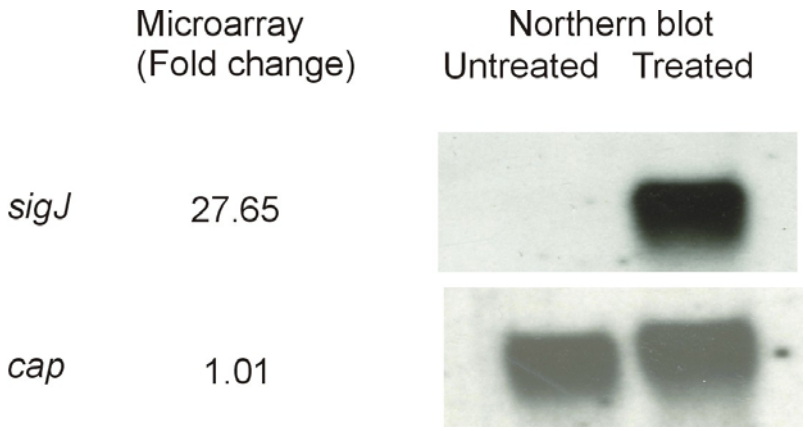

Figure 16. Confirmation of the differential expression of *sigJ* by Northern Blot. Total RNA was extracted from either untreated AX2 cells or after treatment with 200 mM sorbitol for 1 hour. 10 µg RNA was separated under denaturing conditions and Northern blotting was performed as described in material and methods [see 2.2.3.3]. The blot was probed with *sigJ* full length cDNA. *cap* full length cDNA was used as control.

We noted that the absolute values for the fold change that were obtained with real time PCR were in all cases higher than those obtained with the microarray. The Northern blot result also pointed into the same direction. The phenomenon that microarrays often provide compressed measurements in comparison to Northern blots or real time PCR is well known, however, its exact cause is not clear.

3.2.3 Determination of the temporal expression pattern of osmoresponsive genes

Next we analyzed the transcriptional response in a time course experiment. Osmosensing and adaptation are controlled by different mechanisms, and occur at different time scales. For instance, in *E. coli* or Madin-Darby canine kidney cells, K^+ is accumulated within minutes but osmoresponsive genes are expressed within hours or longer after treatment (Kwon and Handler, 1995; Wood, 1999). In *S. cerevisiae*, the induction timing of osmoresponsive genes depends on the severity of the shock (Rep et al., 1999). Based on the research on other organisms and the microarray result described above, the time points 15, 30, 45, 60, 90 and 120 minutes post treatment were chosen for the time course.

For analysis we hybridized six microarrays and thus obtained up to twelve measurements for each probe. To assess the likelihood of falsely reported differentially regulated genes we also compared the transcriptional profile of untreated cells against one another at t_0. Under these conditions, only one gene was reported by SAM without additional threshold (data not shown) and no gene at a threshold of 1.5 (Table 1). This result confirmed the reliability of our analysis pipeline for the detection of differentially regulated genes. Differentially expressed genes were identified throughout the rest of the time course, but their number varied largely. 15 minutes after treatment and at a threshold of 1.5 only 38 genes were identified and, surprisingly, 35 of these were up-regulated. At 30, 45, 60, 90 and 120 minutes 485, 588, 583, 323 and 211 genes were found, respectively, and for all of these time points significantly more genes were down-regulated than up-regulated (Table 1). The largest number of genes was identified 45 and 60 minutes after treatment, when nearly 600 genes were differentially expressed. This corresponds to more than 10% of all spotted probes and indicates that the major

transcriptional changes in *D. discoideum* occur between 30 and 60 minutes after treatment with sorbitol.

Table 1. Number of differentially expressed *Dictyostelium* genes during the two hour time course of sorbitol treatment

Time [min]	Differentially expressed genes		
	Up-regulated	Down-regulated	Sum
0	0	0	0
15	35	3	38
30	219	266	485
45	244	344	588
60	232	351	583
90	120	203	323
120	73	138	211

The expression profiles of the differentially expressed genes were used for a cluster analysis to identify groups of similarly regulated genes. The analysis was performed with GeneSpring 7.2 with a non-redundant set of 809 genes that was created from the 908 regulated genes of the time course. Four major clusters of genes could be identified (Figure 17). The first cluster mainly contained genes that increased in expression throughout the time course with maximal expression at later timepoints, the second one was comprised of genes that were down-regulated between 15 and 60 minutes and then changed to a neutral level or were even slightly up-regulated, the third one was characterized by genes up-regulated mainly between 30 and 60 minutes and the fourth one by genes down-regulated throughout the time course.

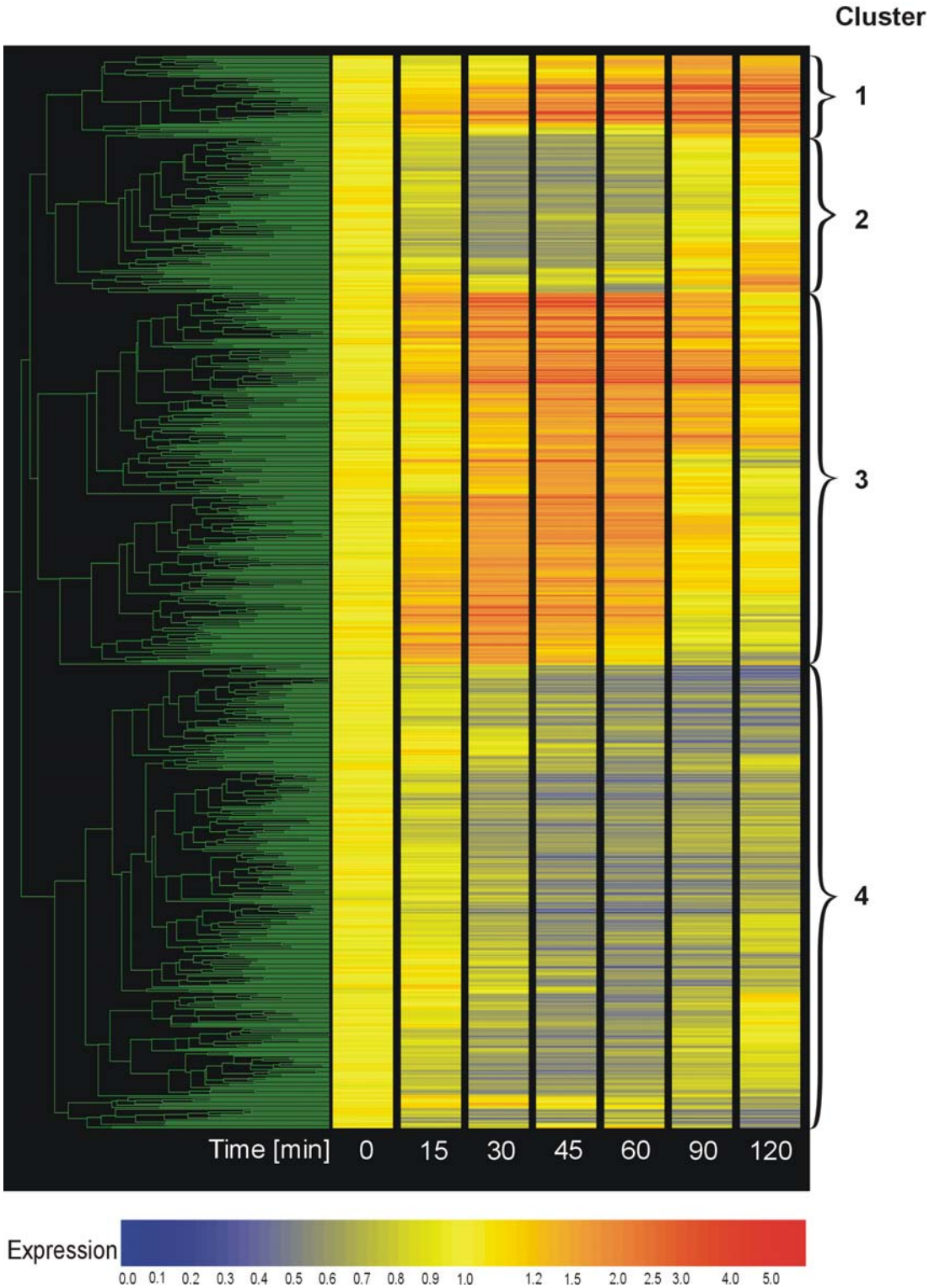

Figure 17. Cluster analysis of differentially regulated genes. A non-redundant set of 809 genes that were up- or down-regulated more than 1.5 fold in the time course of sorbitol treatment were clustered with GeneSpring 7.2. Four major clusters (1-4) can be distinguished. The dendrogram is displayed on the left. The differentially regulated genes are depicted as coloured lines and the time of treatment in minutes is shown at the bottom. The colour represents the fold induction (red) or repression (blue) as shown in the colour scale below the figure. Non-regulated genes are displayed in yellow.

3.2.4 Differentially regulated genes are enriched in distinct functional categories

A common challenge faced by researchers is to translate lists of differentially regulated genes into a better understanding of the underlying biological phenomena. This can be accomplished by the generation of a functional profile that is able to provide insight into the cellular mechanisms relevant in the given condition. The gene ontology (GO; http://www.geneontology.org/) project is an effort to produce a system for annotating gene products that can be applied across all organisms. GO is divided into three categories describing biological processes, molecular functions and cellular components (Harris et al., 2004). GO term enrichment was analyzed with the program GOAT (Xu and Shaulsky, 2005). Only a selection of those biological process GO terms that had a p-value <0.05 are listed (Figure 18, 20, 22 and 24). The full list of all enriched biological process, molecular function and cellular component GO terms is available as supplementary information (Table SI 1).

During data processing we found that only a small fraction of the *Dictyostelium* genes have GO annotations. In addition, some genes were found to be incorrectly annotated, which limited the value of the GO analysis. Therefore we performed in addition manual annotation through database and literature mining, annotated the differentially regulated genes and, based on the yeast classification, classified them into the following 12 groups: 1) Metabolism, 2) Energy, 3) Transcription, 4) Translation, 5) Protein destination (Protein folding and stabilization / proteolysis / protein targeting, sorting and translocation), 6) Cellular biogenesis and organization, 7) Transport, 8) Cell proliferation, 9) Movement/Cytoskeleton, 10) Stress response, 11) Signal transduction, 12) Multicellular organization.

3.2.4.1 Annotation of genes in cluster 1

Analysis with the program GOAT showed on the biological process level an enrichment of genes involved in actin polymerization and/or depolymerization, macromolecule catabolism and proteolysis for cluster 1. Surprisingly, an enrichment of genes involved in culmination during fruiting body formation was also reported. On the cellular component level the proteasome complex was enriched (Figure 18).

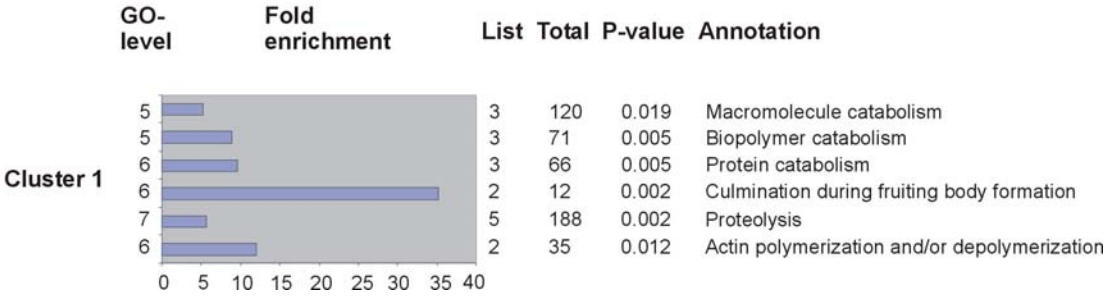

Figure 18. Selection of the GO biological process terms enriched in cluster 1. GO tree levels are shown on the left. Bar lengths represent the fold enrichment (scale x-axis). The table indicates the number of genes with a particular annotation in the cluster (List), on the entire array (Total), the significance for enrichment (P-value) and the annotation.

The categorization of manually annotated genes is shown in table 2. The putative cellobiohydrolase I in the category "metabolism" is highly homologous to the cellobiohydrolase I from *Thermoascus aurantiacus*, which might play a role in the breakdown of carbon sources. Proteasome subunit genes are highly enriched in this cluster as well as other genes involved in proteolysis, for example, the cysteine proteinase 1. Surprisingly, cystatin A3, a putative cysteine protease inhibitor is also found in cluster 1, however it was only slightly up-regulated. We also found a number of genes involved in development and we will consider this interesting finding in more detail in the discussion.

Table 2. Annotation of genes in cluster 1

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
1	metabolism	DDB0231462	***argC***, acetylglutamate kinase, N-acetyl-gamma-glutamyl-phosphate reductase, Ornithine biosynthesis	0.98	1.01	1.04	1.35	1.40	1.67	1.42
		DDB0202169	***dcd2B***, neutral/alkaline nonlysosomal ceramidase family protein. Ceramide metabolism	1.03	1.02	1.00	1.18	1.06	1.25	1.54
		DDB0202233	Cellobiohydrolase I	0.97	1.04	1.40	2.64	3.73	3.49	3.42
5	Protein destination: Protein folding and stabilization / proteolysis / protein targeting, sorting and translocation	DDB0191199	***psmB6***, 20S proteasome subunit beta-6	1.02	0.88	0.96	1.05	1.17	1.54	1.32
		DDB0218287	***psmD11***, 26S proteasome non-ATPase regulatory subunit 11	0.95	0.79	0.84	1.06	1.28	1.53	1.30
		DDB0192101	***psmD2***, 26S proteasome regulatory subunit S2	1.03	0.98	1.18	1.25	1.45	1.51	1.45
		DDB0191435	***psmC4***, 26S proteasome subunit ATPase 4	1.05	0.85	0.92	1.17	1.30	1.55	1.34
		DDB0201647	***cprA***, cysteine proteinase 1	0.95	1.06	1.09	1.12	1.42	2.01	2.31
		DDB0202676	***cpiC***, cystatin A3, putative cysteine protease inhibitor	0.95	1.10	1.23	1.66	1.57	1.67	1.56
		DDB0191154	***cdcD***, cell division cycle protein 48; implicated among other functions in protein degradation	0.89	0.85	0.86	0.95	1.16	1.37	1.56
6	Cellular biogenesis and organization	DDB0169422	***expl3***, expansin-like protein, modify the cell wall to allow expansion during cell growth; expressed in prespore cells	0.99	1.11	1.61	2.42	2.41	2.81	3.28
7	Transport	DDB0219979	***pitB***, phosphatidylinositol transfer protein 2	0.99	0.95	0.88	1.16	1.22	1.74	1.99
9	Movement / Cytoskeleton	DDB0214916	***wdpA***, WD40 repeat protein 2	1.01	0.88	0.93	1.45	1.50	1.65	1.23
		DDB0191444	***mhcA***, myosin II heavy chain	1.00	0.88	0.86	0.92	1.14	1.48	1.64
12	Multicellular organization	DDB0185120	***rtoA***, unknown	0.99	1.10	2.20	4.84	5.02	6.09	5.03
		DDB0215341	***tipD***, homolog of mouse apg16L	1.00	1.22	1.66	1.94	2.02	2.04	2.31
		DDB0185034	***csbA***, contact sites B protein, Cell adhesion	1.02	1.08	1.10	1.43	1.75	2.19	2.36
		DDB0185093	***csbB***, contact sites B protein, Cell adhesion	1.00	1.13	1.16	1.44	1.82	2.03	2.16

Genes that are up-regulated more than 2.0 fold during the time course are labelled in red.

Figure 19 depicts the expression profiles of six genes related to protein degradation. These were first up-regulated 30 to 45 minutes after treatment and for most of them the expression further increased at later time points.

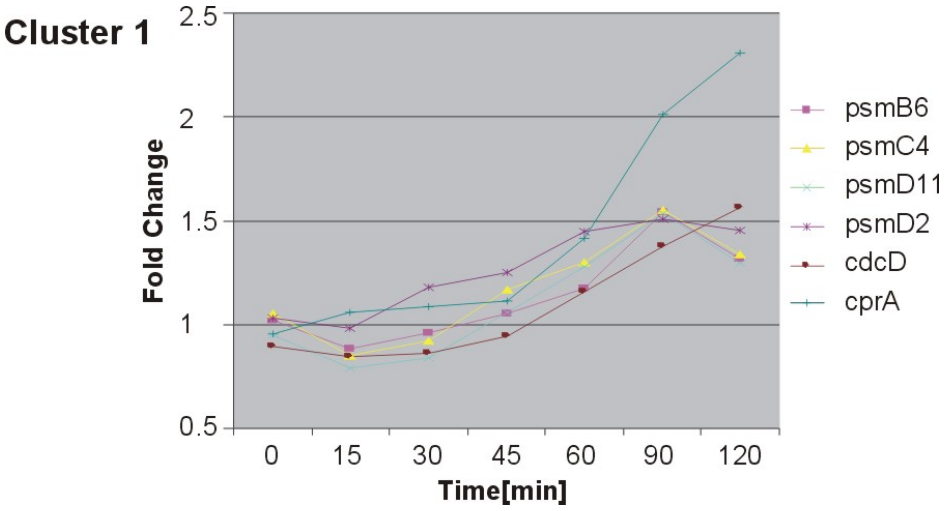

Figure 19. Expression profiles of selected genes from cluster 1.

3.2.4.2 Annotation of genes in cluster 2

Cluster 2 comprises transiently down-regulated genes and shows on the biological process level an enrichment of gene products involved in the response to an external stimulus, in translation and in cellular functions that require cytoskeletal proteins like endocytosis, chemotaxis and cytokinesis (Figure 20). The cellular component category revealed an enrichment of the cortical actin cytoskeleton and this was also reflected in the GO molecular functions where, among others, structural constituents of the cytoskeleton were reported (Table SI 1).

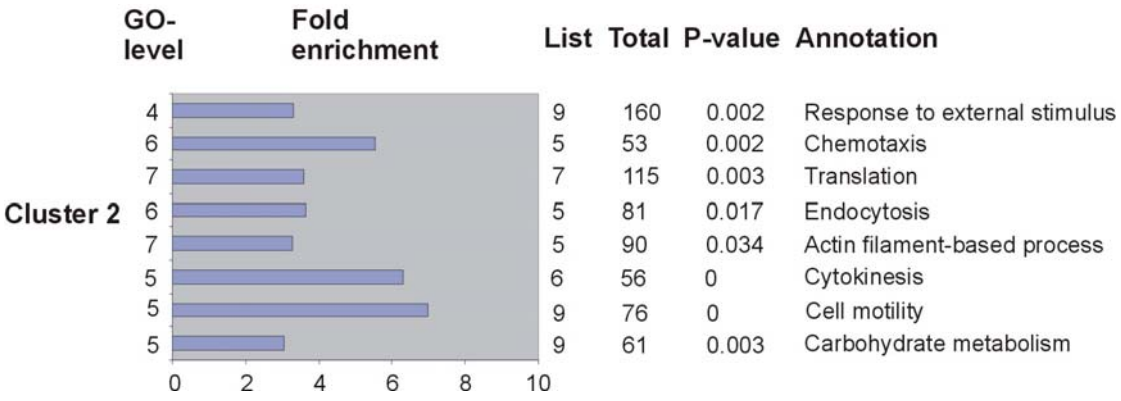

Figure 20. Selection of the GO biological process terms enriched in clusters 2. See figure 18 for figure legend.

The expression profiles of some genes encoding cytoskeletal proteins are shown in Figure 21.

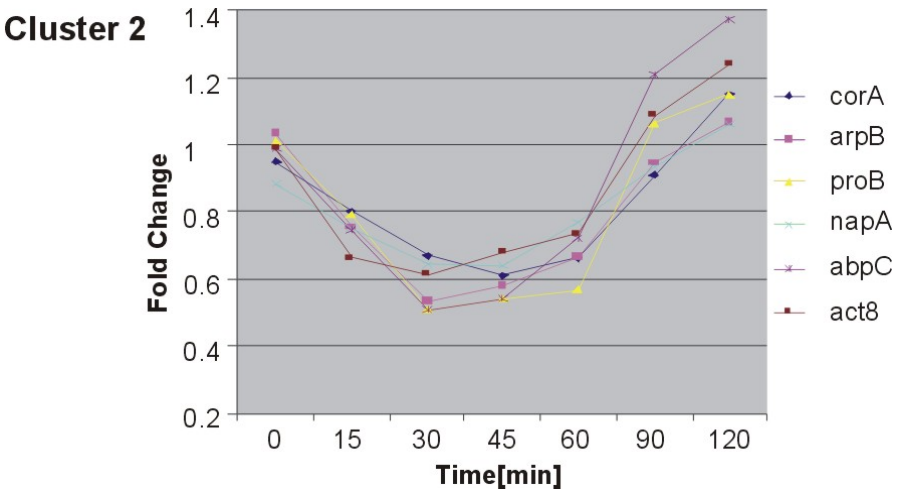

Figure 21. Expression profiles of selected genes from cluster 2.

Manual annotation revealed that most genes reported by GO analysis as "response to external stimulus" are genes encoding cytoskeletal proteins. Manual annotation also showed that several genes grouped into metabolism encode components of the tricarboxylic acid cycle, e.g. *pdhA* (pyruvate dehydrogenase E1 alpha subunit), *pdhX* (pyruvate dehydrogenase complex, component X), *acnB* (aconitate hydratase) and *sdhC* (succinate dehydrogenase), indicating a down-regulation of respiration. In support of this, four genes in the "energy" category (*qinA*, DDB0189294, DDB0216983 and DDB0189366) are NADH-ubiquinone oxidoreductase subunits that act as electron transporter in aerobic respiration. Also the synthesis of new proteins appears down-regulated as we found six translation initiation factors (*eIF3s2*, *eIF1a*, *eIF5b*, *eIF3s6ip*, *moe1* and *eIF4g*) in this cluster. In accordance with the downregulation of cytoskeleton genes, five subunits (*cct2*, *cct8*, *cct3*, *cct7* and *cct5*) of the chaperonin CCT ring complex involved in the folding of actin and tubulin are down-regulated as well. It is noteworthy that a component of the counting factor (CF) complex *cf45-1* and a putative countin receptor *cnrI* are down-regulated [see also 4.2.5].

Table 3. Annotation of genes in cluster 2

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
1	Metabolism	DDB0230185	***bkdB***, branched-chain alpha-keto acid dehydrogenase E1 beta chain, degradation of branched chain amino acids	0.99	0.93	0.65	0.70	0.86	0.96	0.98
		DDB0230168	***acnB***, aconitate hydratase	1.05	0.86	0.50	0.59	0.55	0.87	0.95
		DDB0231385	***sdhC***, succinate dehydrogenase (ubiquinone)	0.95	0.65	0.54	0.55	0.66	0.95	1.10
		DDB0190669	***atp5b***, ATP synthase beta chain, mitochondrial	1.02	0.77	0.57	0.62	0.62	0.79	0.93
		DDB0167552	might be involved in peptidoglycan catabolic process	1.00	1.44	1.07	0.84	0.61	1.29	2.19
		DDB0219864	might be involved in peptidoglycan catabolic process	1.05	1.08	0.81	0.85	0.79	1.22	2.10
		DDB0214943	***glgB***, 1,4-alpha-glucan branching enzyme	0.97	0.92	0.59	0.60	0.58	0.94	1.05
		DDB0187592	Phosphoenolpyruvate carboxylase	1.04	0.90	0.69	0.62	0.76	0.98	1.00
		DDB0214924	***amyA***, putative alpha-amylase	1.00	1.18	0.82	0.81	0.67	1.13	1.59
		DDB0230193	***pdhA***, pyruvate dehydrogenase E1 alpha subunit	1.03	0.91	0.65	0.68	0.71	0.91	0.90
		DDB0230192	***pdhX***, pyruvate dehydrogenase complex	1.09	0.78	0.63	0.64	0.72	0.93	0.95
		DDB0216178	***sgmA***, sphingomyelinase, ceramide biosynthesis	1.03	0.86	0.76	0.62	0.72	1.02	1.21
		DDB0235198	***mecr***, trans-2-enoyl-CoA reductase, fatty acid biosynthetic process	0.90	0.83	0.69	0.61	0.84	0.96	1.18
		DDB0167445	***fahd1***, Fumarylacetoacetate (FAA) hydrolase domain-containing protein	0.99	0.92	0.70	0.65	0.75	0.85	0.94
		DDB0169464	acyl-CoA oxidase	0.98	0.82	0.63	0.62	0.64	0.86	1.14
		DDB0187716	Formimidoyltransferase-cyclodeaminase (FTCD), Includes Glutamate formyltransferase	0.95	0.82	0.77	0.61	0.73	0.97	1.14
		DDB0231397	***fumH***, fumarate hydratase	1.02	0.90	0.69	0.65	0.79	1.03	1.04
		DDB0188841	Short-chain oxidoreductase.	0.95	1.01	0.85	0.73	0.73	1.18	1.67
		DDB0214894	***alaS***, alanyl-tRNA synthetase	0.99	0.93	0.84	0.66	0.81	1.19	1.28
		DDB0231305	***serS***, seryl-tRNA synthetase	0.96	0.77	0.54	0.54	0.65	1.00	1.20
		DDB0231245	***trpS***, tryptophanyl-tRNA synthetase	0.95	0.79	0.61	0.59	0.62	1.00	1.05
2	Energy	DDB0189294	NADH dehydrogenase (ubiquinone)	0.99	0.78	0.61	0.70	0.73	0.94	0.97
		DDB0216983	NADH dehydrogenase (ubiquinone)	0.98	0.73	0.59	0.76	0.74	0.90	1.01
		DDB0191420	***qinA***, ubiquinone oxidoreductase	0.98	0.82	0.65	0.68	0.70	0.99	1.04
		DDB0189366	putative NADH dehydrogenase (ubiquinone)	0.97	0.80	0.63	0.67	0.72	1.00	1.11
		DDB0167789	***vatH***, vacuolar ATP synthase subunit H	0.99	0.88	0.55	0.57	0.56	0.77	1.10
		DDB0201593	***cytB***, cytochrome b	1.12	0.89	0.67	0.69	0.65	0.85	0.92
3	Transcription	DDB0215406	***rpb1***, RNA polymerase II largest subunit	0.97	0.85	0.59	0.64	0.75	1.00	1.11
		DDB0205969	***snd1***, (SNase-like) domain-containing protein, regulation of transcription from RNA polymerase II promoter	0.99	0.79	0.66	0.69	0.77	1.00	1.12
4	Translation	DDB0186657	***eIF3s2***, eIF-3 beta	1.04	0.87	0.65	0.68	0.61	1.10	1.15
		DDB0204504	***eIF1a***, eukaryotic translation initiation factor 1A	1.00	0.77	0.58	0.80	0.93	1.17	1.27
		DDB0206214	***eIF5b***, eukaryotic translation initiation factor 5B	1.01	0.91	0.64	0.63	0.68	0.94	1.00
		DDB0168640	***eIF3s6ip***, eukaryotic translation initiation factor 3 (eIF3) subunit 6 interacting protein	0.95	0.81	0.61	0.62	0.75	1.01	1.07
		DDB0219927	***moe1***, eIF-3 zeta	1.01	0.78	0.56	0.54	0.64	0.90	0.96
		DDB0217646	***eIF4g***, eukaryotic translation initiation factor 4 gamma	1.05	0.79	0.53	0.59	0.72	0.89	0.96
		DDB0231065	***rps27***, 40S ribosomal protein S27	0.99	0.85	0.66	0.65	0.79	1.03	1.07
5	Protein destination: Protein folding and stabilization / proteolysis / protein targeting, sorting and translocation	DDB0218815	***pfdn3***, prefoldin alpha-like domain containing protein	1.05	0.80	0.65	0.84	0.81	1.04	1.07
		DDB0188902	FKBP-type peptidylprolyl cis-trans isomerase domain-containing protein	1.03	0.95	0.59	0.53	0.62	0.75	1.00
		DDB0183841	***cct2***, chaperonin containing TCP1 beta subunit	1.08	0.84	0.57	0.67	0.70	0.90	0.94
		DDB0217758	***cct8***, chaperonin containing TCP1 theta subunit	0.98	0.79	0.61	0.63	0.66	0.94	1.06
		DDB0204641	***cct3***, chaperonin containing TCP1 gamma subunit	1.03	0.77	0.54	0.58	0.74	0.93	1.01
		DDB0191096	***cct7***, chaperonin containing TCP1 eta subunit	0.93	0.88	0.65	0.58	0.71	0.93	1.06
		DDB0204244	***cct5***, chaperonin containing TCP1 epsilon subunit	0.98	0.77	0.60	0.65	0.74	1.14	1.20
		DDB0191107	***fpaA***, cytosolic glycoprotein FP21	0.97	0.69	0.60	0.58	0.69	0.93	1.05
		DDB0185043	***fpaB***, cytosolic glycoprotein FP21	0.98	0.59	0.49	0.48	0.66	0.98	1.18
		DDB0191291	***lkhA***, leukotriene A4 hydrolase	1.04	0.79	0.61	0.62	0.72	0.99	1.13
		DDB0169154	putative ubiquitin-conjugating enzyme E2	1.03	0.83	0.65	0.80	0.76	1.01	0.99

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
6	Cellular biogenesis and organization	DDB0191831	***ost1***, dolichyl-diphosphooligosaccharide-protein glycotransferase	0.97	0.74	0.55	0.61	0.68	0.99	1.07
		DDB0219627	***ost2***, dolichyl-diphosphooligosaccharide-protein glycotransferase	1.01	0.83	0.60	0.65	0.71	0.90	1.01
		DDB0191760	telomerase-associated protein 1-like protein, involved in replication of chromosome termini	1.09	0.92	0.70	0.56	0.60	1.19	1.53
		DDB0215348	***cnxA***, calnexin, Ca2+-binding protein with chaperone activity in the endoplasmic reticulum	1.01	0.73	0.59	0.64	0.73	0.91	0.96
7	Transport	DDB0201663	***nutf2***, nuclear transport factor 2	1.07	0.79	0.64	0.63	0.63	0.85	1.05
		DDB0191505	***vacA***, vacuolin A	0.95	0.73	0.64	0.65	0.62	0.80	1.14
		DDB0205708	***CAX1***, transports Ca2+ or other cations using the gradient of H+ or Na+ generated by energy-coupled primary transporters	0.94	1.03	0.79	0.83	0.80	0.96	1.69
		DDB0187628	***sec61g***, protein transport protein SEC61 gamma subunit	1.01	0.67	0.52	0.58	0.62	0.80	0.91
		DDB0191102	***apm1***, clathrin-adaptor medium chain apm1, hypotonic response	1.00	0.85	0.65	0.89	0.89	1.09	1.05
8	Cell proliferation	DDB0191488	***zipA***, zipper-like domain-containing protein	1.00	0.72	0.63	0.64	0.79	0.94	0.94
9	Movement / cytoskeleton	DDB0191115	***corA***, actin binding protein	0.95	0.80	0.67	0.61	0.66	0.91	1.15
		DDB0220444	***act1***, actin	0.83	0.71	0.57	0.60	0.67	1.22	1.13
		DDB0185015	***act15***, actin	0.84	0.73	0.57	0.58	0.62	1.27	1.13
		DDB0216213	***act8***, actin	0.99	0.66	0.61	0.68	0.74	1.09	1.24
		DDB0191249	***proB***, profilin II	1.01	0.79	0.51	0.54	0.57	1.06	1.15
		DDB0185179	***arpB***, actin related protein 2	1.03	0.76	0.53	0.58	0.67	0.95	1.07
		DDB0231423	***napA***, component of SCAR regulatory complex	0.89	0.75	0.64	0.64	0.77	0.93	1.06
		DDB0201554	***abpC***, gelation factor	0.99	0.75	0.51	0.54	0.72	1.21	1.37
		DDB0214810	***abpB***, actin binding protein	0.97	0.57	0.51	0.49	0.68	1.16	1.31
		DDB0214939	***limE***, LIM domain-containing protein	0.98	0.69	0.49	0.44	0.47	0.90	1.12
		DDB0203397	putative actin binding protein	1.03	0.71	0.67	0.96	1.22	1.66	1.30
11	Signal transduction	DDB0191337	***mvpB***, major vault protein	0.99	0.81	0.52	0.46	0.57	0.82	1.07
		DDB0201663	***RasG***, protein kinase B related	1.07	0.79	0.64	0.63	0.63	0.85	1.05
		DDB0191476	***rab1A***, Rab GTPase	1.02	0.71	0.58	0.74	0.77	1.03	1.03
		DDB0191161	***cf45-1***, component of the counting factor (CF) complex	1.08	1.11	0.84	0.71	0.57	0.81	1.34
		DDB0229864	***cnrl***, putative countin receptor Cnr9	1.02	0.90	0.57	0.66	0.63	0.85	0.98
12	Multicellular organization	DDB0186637	TipA	1.00	0.64	0.65	0.73	0.80	0.92	0.99

Genes that are down-regulated more than 2.0 fold during the time course are labelled in blue.

3.2.4.3 Annotation of genes in cluster 3

The GO analysis for the up-regulation genes of cluster 3 revealed on the biological process level an enrichment of genes involved in the response to oxidative stress, in late endosome to vacuole transport, in the G1/S transition of the mitotic cycle and in development, in particular culmination during fruiting body formation and sporulation. In addition, the GO molecular function terms showed for this cluster an enrichment of genes encoding transporters, transcriptional repressors, Ras GTPase activators and inhibitors, Ser/Thr protein kinases, Rho GTPase binding proteins and cytoskeletal proteins (Figure 22 and Table SI 1 for details).

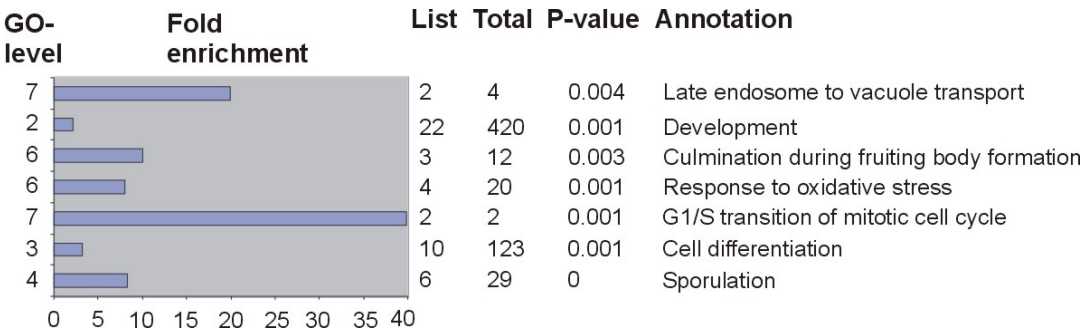

Figure 22. Selection of the GO biological process terms enriched in clusters 3. See figure 16 for figure legend.

The expression profiles of genes assigned to the biological process category "response to oxidative stress", STATc (*dst*C), RasGAP (*gap*A), severin kinase (*svk*A) and an ABC transporter (*abc*G21) are depicted in Figure 23. We will consider *Dictyostelium* STATc in more detail below.

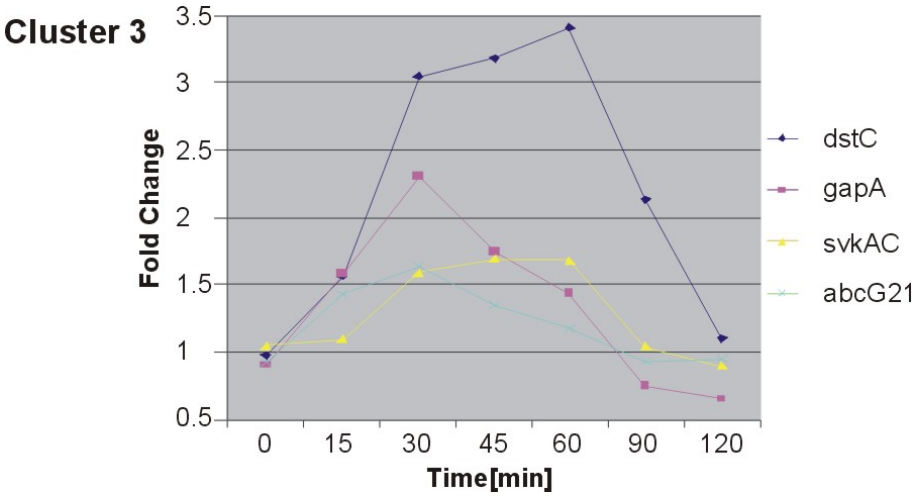

Figure 23. Expression profiles of selected genes from cluster 3.

Manual annotation revealed a cluster of genes involved in the degradation of AMP and urate, e.g. *amdA* (AMP deaminase), *DDB0231470* (uricase) and *allC* (allantoicase). This process results in the generation of urea and glyoxylate. Some genes are involved in the breakdown of carbon sources, e.g. *celA* (cellulase), *celB* (cellulose binding protein) and *DDB0204945* (alpha amylase family protein), others in the biosynthesis of trehalose, e.g. *tpsA* (treholase 6-phosphate synthase), and in the biosynthesis of proline, e.g. *DDB0190241* (pyrroline-5-carboxylate reductase). Similar to results in cluster 1 we found a large number of genes, around 20, involved in proteolysis,

e.g. *culA* (cullin), *ubqA* (ubiquitin), and *ubqD* (ubiquitin). The up-regulation of these genes might lead to the accumulation of organic solutes, which could be utilized by *Dictyostelium* as osmolytes [see 3.2.5 and 4.2.2]. Transcription factors are especially interesting because they could be responsible for the differential regulation of target genes. Beside STATc (*dstC*), we find five other genes encoding transcription factors, e.g. *stkA* (STalKy mutant), *srfA* (serum response factor A), *dstA*, *dstB* and *repB* (involved in DNA repair) in this cluster. Transporter genes are also prominent members of this cluster, e.g. *abcB1* (ABC transporter), *abcG21* (ABC transporter), *DDB0189650* (a sodium/potassium-transporting ATPase), and three mitochondrial substrate carriers. Impressively in total 11 cytoskeletal genes are up-regulated, which could play roles in reorganization of the cytoskeleton. In the signal transduction category, genes encoding small GTPases and PH domain containing proteins as well as a number of protein kinases are found.

Table 4. Annotation of genes in cluster 3

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
1	Metabolism	DDB0191089	***amdA***, AMP deaminase, purine degradation	0.94	1.87	3.91	3.39	2.43	0.78	0.75
		DDB0185186	***gnt2***, GlcNAc transferase, mycothiol biosynthesis	0.94	1.07	1.37	1.45	1.60	1.22	0.98
		DDB0231436	glutathione S-transferase domain-containing protein	1.04	1.29	1.40	1.53	1.29	1.03	1.02
		DDB0231471	***allC***, allantoicase, urate degradation	0.98	1.52	3.10	4.89	4.74	3.47	1.99
		DDB0231470	uricase, urate degradation	1.01	0.92	1.27	1.94	2.08	1.83	1.11
		DDB0231393	acyl-CoA oxidase	0.98	1.16	1.53	1.55	1.50	1.08	1.02
		DDB0167310	putative oxidoreductase, oxygen dependent, fad-dependent protein	0.99	1.34	1.52	1.39	1.37	1.13	1.11
		DDB0188682	***grxA***, glutaredoxin	1.03	1.25	1.40	1.52	1.53	1.28	1.05
		DDB0230093	***aatB***, aspartate aminotransferase, aspartate degradation	1.00	1.15	1.46	1.64	1.68	1.60	1.29
		DDB0190241	pyrroline-5-carboxylate reductase, proline biosynthesis	0.94	1.72	2.15	2.56	2.59	2.42	2.02
		DDB0219248	***tpsA***, glycosyltransferase, Treholose biosynthesis	1.01	0.90	1.28	2.24	2.89	3.00	2.06
		DDB0218638	***aass***, aminoadipic semialdehyde synthase, NAD+, L-glutamate-forming	1.00	1.35	1.68	1.47	1.30	0.87	0.78
		DDB0185328	***sodB***, superoxide dismutase	0.99	1.54	2.32	2.08	2.30	2.23	1.96
		DDB0203727	antioxidant enzyme	0.88	2.29	3.80	5.52	6.09	5.85	3.36
		DDB0191537	***ugt52***, sterol glucosyltransferase	0.97	1.16	1.71	2.29	1.86	1.03	0.86
		DDB0214890	***SRE1***, steroid isomerase	0.97	1.37	1.70	2.15	2.35	2.05	1.26
		DDB0217424	CABP1-related protein	0.96	0.94	1.01	1.36	1.72	1.30	1.20
		DDB0188646	Serine:pyruvate/alanine:glyoxylate aminotransferase	0.98	1.23	1.54	1.72	1.58	1.35	1.03
		DDB0229908	***acnA***, putative iron regulatory protein	1.01	0.91	1.10	1.23	1.52	1.36	1.16
		DDB0204945	Alpha amylase family protein, oligosaccharide degradation	0.92	1.55	2.90	4.04	3.92	2.52	1.40
		DDB0205008	Acetylornitine deacetylase, lysine biosynthesis	0.96	1.18	1.49	1.76	1.61	1.55	1.37
		DDB0215351	***celA***, cellulose binding protein, cellulase	0.86	1.18	1.78	1.64	1.42	1.19	1.07
		DDB0191122	***celB***, cellulose binding protein	0.93	1.96	3.83	5.59	4.08	2.11	1.39
		DDB0187034	glutamine-fructose-6-phosphate transaminase	0.93	1.14	1.47	1.52	1.54	1.19	0.93
		DDB0168688	3-hydroxyacyl-CoA dehydrogenase type II	0.99	1.00	1.27	1.75	1.81	1.58	1.44
		DDB0186041	***fut5***, glycosyltransferase, mycothiol biosynthesis	1.00	1.24	1.56	1.75	1.56	0.96	0.90
		DDB0187554	***mpgA***, mannose-1-phosphate guanylyltransferase	1.03	1.09	1.37	1.54	1.33	1.04	1.04

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
3	Transcription	DDB0185187	***stkA***, GATA Zn finger-containing protein	1.17	1.10	1.53	1.73	1.48	0.98	0.98
		DDB0214892	***srfA***, MADS-box transcription factor	0.84	1.08	1.56	1.14	1.30	0.98	1.05
		DDB0215388	***dstA***, STAT family protein	0.97	1.18	1.55	1.40	1.28	1.05	0.96
		DDB0215378	***dstC***, STATfamily protein	0.98	1.57	3.05	3.18	3.41	2.13	1.11
		DDB0191116	***dstB***, STATfamily protein	1.00	1.26	2.04	1.93	1.74	0.99	0.89
		DDB0214830	***repB***, XPB, transcription factor IIH subunit	1.04	1.01	1.20	1.58	1.56	1.13	1.00
4	Translation	DDB0201621	***mrps2***, ribosomal protein S2	0.93	1.16	1.50	1.27	1.10	0.91	0.62
		DDB0201604	***mrps7***, ribosomal protein S7	0.92	1.44	1.69	1.03	0.88	0.84	0.81
		DDB0201607	***DidioMp26***, ribosomal protein S3, N-terminal domain	1.07	1.68	2.01	1.15	0.90	0.67	0.62
		DDB0201608	***DidioMp27***, ribosomal protein S3, C-terminal domain	0.83	1.43	1.96	1.35	1.06	0.69	0.54
		DDB0187131	***eIF5***, eukaryotic translation initiation factor 5	0.99	1.45	2.07	2.04	1.77	1.23	1.14
5	Protein destination: Protein folding and stabilization / proteolysis / protein targeting, sorting and translocation	DDB0216177	***dymA***, dynamin like protein	0.98	1.07	1.60	1.73	1.71	1.14	1.07
		DDB0185191	***culA***, cullin	0.97	1.05	1.33	1.71	1.55	1.23	1.08
		DDB0186248	Putative cullin 3.	1.00	1.13	1.52	1.25	1.44	1.00	1.01
		DDB0191171	***cplA***, calpain-like cysteine protease	1.00	1.06	1.57	1.94	2.00	1.23	0.72
		DDB0219849	Putative FtsH protease	1.03	0.97	1.27	1.51	1.53	1.04	0.98
		DDB0187705	***psmD1***, 26S proteasome regulatory subunit S1	0.92	1.13	1.22	1.24	1.61	1.35	1.24
		DDB0191298	***psmD14***, 26S proteasome non-ATPase regulatory subunit 14	0.95	0.95	1.22	1.46	1.55	1.33	1.12
		DDB0205381	SppA, signal peptidase activity	0.93	1.02	1.30	1.55	1.50	1.24	0.90
		DDB0220657	***cpiA***, cystatin A1, proteolysis inhibitor	1.01	1.22	1.53	1.67	1.55	1.61	1.50
		DDB0191056	ubiquitin-protein ligase activity	1.02	1.30	1.81	1.41	1.25	0.91	0.89
		DDB0214921	***ubqA***, ubiquitin	0.80	1.21	1.71	1.99	2.40	1.24	0.63
		DDB0201651	***ubqF***, ubiquitin	0.94	1.14	1.38	1.92	1.70	1.02	0.71
		DDB0214920	***ubqG***, ubiquitin precursor	0.89	1.48	1.89	2.24	2.24	1.11	0.77
		DDB0214929	***ubqD***, ubiquitin	0.95	1.27	1.69	2.05	1.90	1.08	0.86
		DDB0202821	***usp14***, peptidase C19 family protein, contain ubiquitin domain	1.00	0.96	1.24	1.67	1.97	1.95	1.50
		DDB0183881	***ubpA***, deubiquitinating enzyme	0.90	1.19	1.81	2.18	1.99	1.49	1.00
		DDB0215686	***CSN1***, COP9 signalosome complex subunit 1	1.02	1.04	1.24	1.56	1.50	1.33	1.11
		DDB0190801	ZZ-type Zn finger-containing protein, ubiquitin-associated (UBA) domain-containing protein	0.99	0.99	1.53	1.86	1.92	1.27	0.83
		DDB0191418	***rbrA***, ariadne-like ubiquitin ligase	1.01	1.23	1.74	1.94	2.03	1.36	0.98
		DDB0188049	ARIADNE-like protein ARI2, protein ubiquitination	0.98	1.38	1.79	1.36	1.25	1.07	0.97
		DDB0204785	***prlA***, proliferation associated protein, highly conserved metalloexopeptidase, proteolysis	1.01	1.30	1.46	1.65	1.77	1.56	1.17
6	Cellular biogenesis and organization	DDB0191317	***vps46***, SNF7 family protein	0.96	1.23	1.72	1.88	1.93	1.31	0.94
		DDB0231531	***vps32***, SNF7 family protein, involved in vesicle trafficking in yeast and mammals; expressed in endosome	1.04	0.80	1.06	1.60	1.55	1.09	0.95
		DDB0206451	***vps55***, vacuolar protein sorting 55 family protein	1.01	0.97	1.16	1.73	1.56	1.20	0.97
		DDB0191112	***tipC***, vacuolar protein sorting-associated protein	0.98	1.18	1.50	1.94	1.87	1.56	1.19
		DDB0203662	vacuolar sorting protein 9 domain-containing protein	1.00	1.10	1.60	1.38	1.21	0.88	0.84
		DDB0185960	***vps4***, AAA ATPase domain-containing protein	0.95	0.94	1.29	1.64	1.78	1.49	1.17
		DDB0169544	***sf3b1***, splicing factor 3B subunit 1	1.05	1.33	2.02	2.19	2.08	1.34	1.21
		DDB0189279	RNA-binding region-containing protein (RNP-1)	1.05	1.15	1.57	1.35	1.52	1.27	1.06
		DDB0220666	SAP DNA-binding domain-containing protein, RNA-binding region-containing protein (RNP-1), RNA recognition motif-containing protein (RRM)	0.87	1.19	1.54	1.27	1.38	0.96	0.97
		DDB0169252	Mitochondrial genome maintenance protein MGM101 precursor.	1.00	1.23	1.59	2.09	1.94	1.72	1.25
7	Transport	DDB0185907	***slc25a11***, mitochondrial 2-oxoglutarate/malate carrier	0.98	1.28	1.85	2.41	2.16	1.11	1.01
		DDB0217773	mitochondrial substrate carrier family protein	0.95	1.46	2.32	2.43	2.00	1.39	1.11
		DDB0191266	***mftA***, mitochondrial substrate carrier family protein	0.99	1.21	1.47	1.54	1.38	0.89	0.87
		DDB0189650	sodium/potassium-transporting ATPase alpha chain 2	1.03	1.71	3.24	3.09	3.15	1.36	0.87
		DDB0191239	***abcG21***, ABC transporter G family protein	0.93	1.43	1.64	1.35	1.18	0.93	0.95
		DDB0201666	***abcB1***, ABC transporter B family protein	1.10	1.98	2.92	3.32	2.80	1.63	1.05
		DDB0185587	Zinc transporter.	0.96	0.91	1.08	1.55	1.49	1.09	0.95
		DDB0206553	Putative impotin alpha 1b	0.96	1.12	1.44	1.57	1.82	1.38	1.07
		DDB0214946	***patB***, P-type ATPase	1.03	0.91	1.62	1.97	2.24	1.84	1.24
		DDB0188438	P-type ATPase	1.07	1.07	1.23	1.81	1.35	1.27	1.18
		DDB0215359	***clcA***, chloride channel protein, chloride transport	0.98	1.37	1.58	1.32	1.44	0.98	0.99
		DDB0215368	***cpnA***, phospholipid-binding protein	1.00	0.89	1.08	1.57	1.40	1.08	0.90

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
9	Movement / cytoskeleton	DDB0191103	***ctxA***, actin binding protein	0.98	1.14	1.68	2.01	2.30	1.78	1.20
		DDB0185031	***ctxB***, cortexillin II	1.01	1.10	1.57	1.71	1.82	1.35	0.87
		DDB0232318	***slaA***, putative actin binding protein	0.98	1.25	1.82	2.20	2.10	1.34	0.85
		DDB0214996	***forA***, actin binding protein	1.06	1.12	1.56	1.96	1.77	1.39	1.12
		DDB0231185	***forF***, actin binding protein	1.05	0.93	1.22	1.52	1.45	0.99	0.91
		DDB0185086	***myoK***, myosin IK	0.94	1.47	2.09	1.95	2.05	1.36	0.98
		DDB0215392	***myoA***, myosin IA heavy chain	1.07	1.06	1.14	1.39	1.77	1.68	1.15
		DDB0191252	***dct***, dynacortin	0.94	1.47	2.04	2.15	2.26	1.69	1.29
		DDB0216663	***abnA***, actobindin	1.00	1.35	1.76	1.95	1.72	1.66	1.63
		DDB0190330	***abnB***, actobindin	0.97	1.29	1.73	1.91	1.71	1.57	1.32
		DDB0206583	***ksnG***, K7 kinesin-like protein, microtubule motor activity	0.96	1.16	1.90	2.26	2.14	1.26	0.98
10	Stress response	DDB0185022	***capA***, cAMP-binding protein	1.00	1.03	1.23	1.48	1.96	1.52	1.31
		DDB0185023	***capB***, CABP1-related protein	0.99	1.02	1.30	1.32	1.51	1.16	1.07
11	Signal transduction	DDB0191437	***rgaA***, RasGTPase-activating protein	0.98	1.14	1.53	1.70	2.00	1.51	1.19
		DDB0229433	***rasX***, Ras GTPase	1.13	1.23	1.55	1.48	1.40	0.79	0.82
		DDB0229402	***rab2B***, Rab GTPase	0.99	1.40	1.80	2.07	1.59	1.13	1.03
		DDB0203974	***gxcEE***, RhoGEF domain-containing protein	0.95	1.23	1.51	1.48	1.62	1.56	1.40
		DDB0214826	***racI***, Rho GTPase	1.02	1.29	1.62	1.78	1.85	1.35	1.01
		DDB0191293	***gapA***, RasGTPase-activating protein	0.91	1.58	2.31	1.74	1.44	0.76	0.65
		DDB0215004	***gefC***, regulator of chromosome condensation (RCC1) domain-containing protein	1.04	1.32	1.67	1.57	1.29	1.05	1.08
		DDB0202154	***gxcGG***, pleckstrin homology (PH) domain-containing protein	1.00	1.09	1.60	1.53	1.25	0.96	0.88
		DDB0188911	***gxcHH***, pleckstrin homology (PH) domain-containing protein	0.95	1.03	1.28	1.50	1.30	1.04	1.05
		DDB0169060	***gxcBB***, pleckstrin homology (PH) domain-containing protein	1.04	1.46	1.99	1.84	1.63	1.03	0.86
		DDB0191767	DOCK family protein, gef activity	0.97	1.13	1.58	1.66	1.81	1.18	0.92
		DDB0233081	***xpr1***, putative xenotropic and polytropic retrovirus receptor, also similar to yeast SYG1, a G-protein associated signal transduction protein	1.07	1.53	2.60	3.24	2.85	1.39	1.04
		DDB0217181	Cyclic AMP receptor 1	0.95	1.70	1.80	1.08	0.90	0.78	0.84
		DDB0231199	MORN repeat-containing protein kinase	1.07	1.46	1.96	1.89	1.44	0.94	0.87
		DDB0229845	***phg2***, protein serine/threonine kinase	0.90	1.36	1.75	1.23	1.31	1.05	0.96
		DDB0229344	putative protein serine/threonine kinase	0.97	1.00	2.04	1.91	1.75	0.87	0.61
		DDB0219947	***ndrB***, putative protein serine/threonine kinase	1.00	1.36	2.00	1.88	1.46	1.10	1.05
		DDB0216387	putative protein serine/threonine kinase	1.04	1.22	1.61	1.42	1.31	0.99	0.97
		DDB0216346	putative casein kinase II regulatory subunit	1.03	1.37	1.77	1.40	1.16	0.95	0.95
		DDB0191218	***pkyA***, protein tyrosine kinase	0.96	0.97	1.36	2.00	1.78	1.52	1.16
		DDB0191176	***svkA***, severin kinase	1.05	1.10	1.59	1.69	1.68	1.04	0.90
		DDB0191155	***cdk5***, protein serine/threonine kinase	1.03	1.11	1.34	1.70	1.63	1.37	1.08
		DDB0216190	***ppkA***, polyphosphate kinase	1.00	1.05	1.44	1.86	1.88	1.43	1.18
		DDB0215670	***splA***, non-receptor tyrosine kinase	1.05	1.07	1.49	1.64	1.43	1.10	0.95
		DDB0230051	Zn finger-containing protein, putative protein serine/threonine kinase	1.00	1.22	1.65	1.53	1.37	1.10	1.14
		DDB0214986	***ptpC***, protein tyrosine phosphatase	0.95	1.39	2.06	2.21	1.78	1.06	0.85
		DDB0185021	***canA***, calcineurin A, protein serine/threonine phosphatase, protein phosphatase-2B	1.00	1.10	1.35	1.58	1.61	1.39	1.22
		DDB0229894	***fcpA***, putative CTD phosphatase	1.00	1.85	2.98	3.17	3.28	2.31	1.45
		DDB0191353	***sigI***, CBS (cystathionine-beta-synthase) domain-containing protein, may play a regulatory role for other proteins	0.97	1.08	1.82	2.09	3.30	2.08	1.33
12	Multicellular organization	DDB0220027	***culD***, culmination specific protein 45D	0.94	1.16	2.38	2.19	2.53	2.27	1.68
		DDB0185953	putative glycoside hydrolase	0.98	1.22	1.63	1.57	1.47	1.08	1.06

See table 2 for figure legend.

3.2.4.4 Annotation of genes in cluster 4

As expected, the lists of enriched GO terms for the mainly down-regulated genes in cluster 4 differed considerably from cluster 1 and 3. Cluster 4 is characterized by down-regulated genes, which either remained repressed throughout the time course or returned to normal levels at the end of the two hours treatment. On the biological process level genes whose products are involved in all aspects of metabolism were enriched indicating that the cells reduced their metabolic activities upon exposure to high osmolarity (Figure 24).

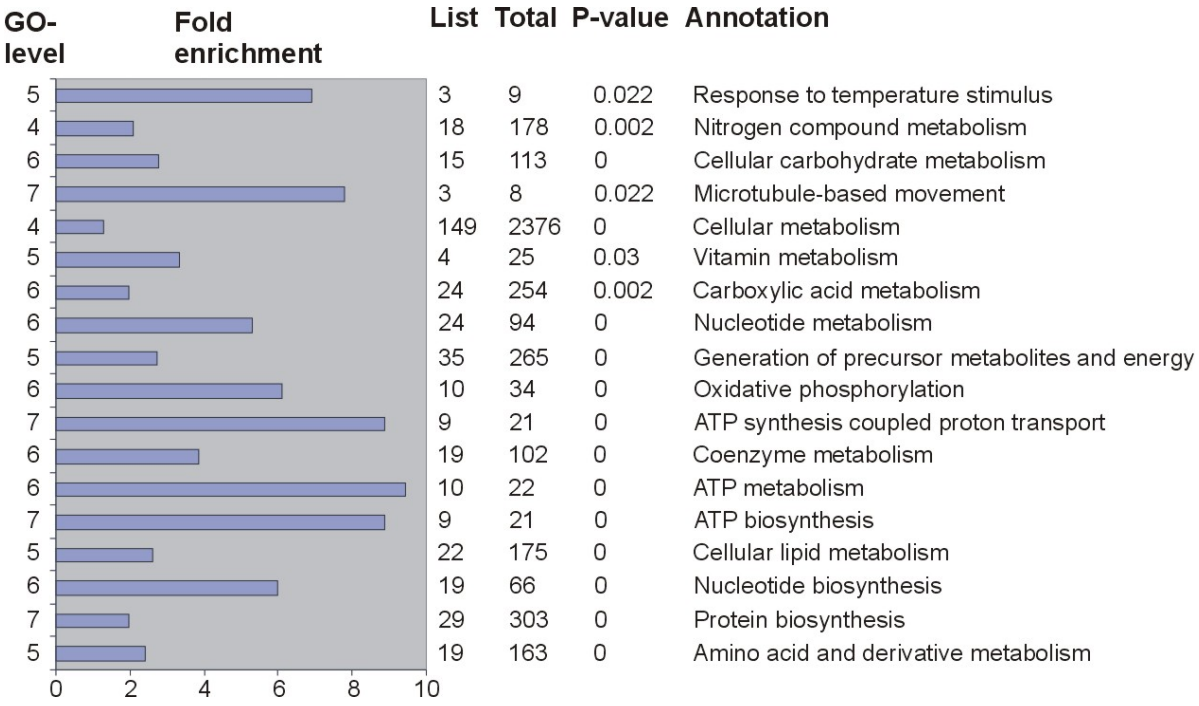

Figure 24. Selection of the GO biological process terms enriched in clusters 4. See figure 18 for figure legend.

Interestingly, Manual annotation showed that all genes encoding the different subunits of the vacuolar ATPase were regulated in a very similar manner (Figure 25).

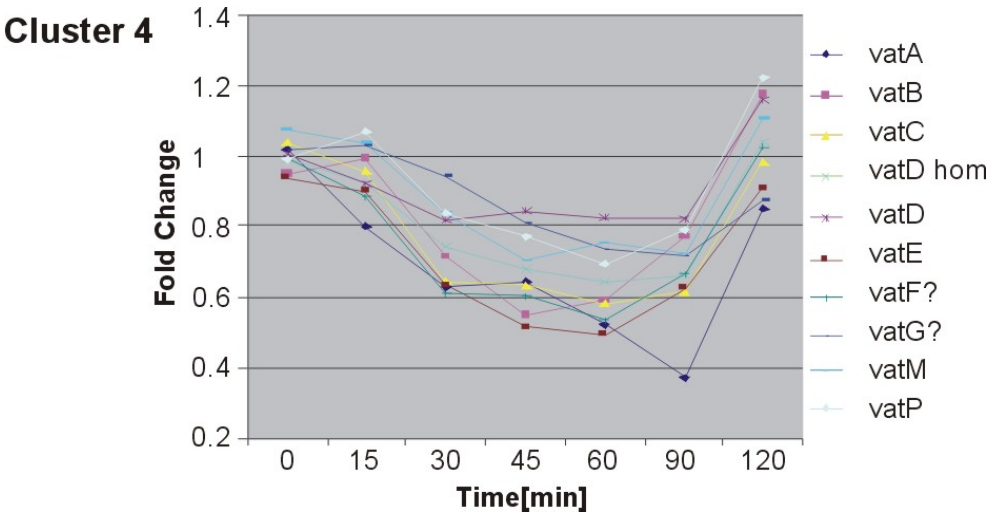

Figure 25. Expression profiles of selected genes from cluster 4. The question mark refers to vatF and vatG being putative vatF and vatG

Many genes involved in amino acid, purine and pyrimidine metabolism are present in this cluster. In the "energy" category not as many NADH dehydrogenases were found as in cluster 2, but more cytochrome genes were present, e.g. *cytC* (cytochrome c), *cxdA* (cytochrome c oxidase subunit IV), *cxeA* (cytochrome c oxidase subunit IV). In the "transcription" category, the gene encoding RNA polymerase I subunit is repressed throughout the 2 hour time course, while the RNA polymerase II subunit in cluster 2 returned to normal levels at later time points. Concomitantly 22 genes encoding ribosomal proteins or ribosomal RNA as well as translation elongation factors are present in this cluster. We also find a number of genes encoding cytoskeletal proteins in this cluster, e.g. *comA* (comitin), *forB* (formin homology domain-containing protein), *hatB* (hisactophilin II), *ponA* (ponticulin). In the "signal transduction" category, two genes, *ctnA* (countin) and *smlA* (smallA), which regulating the size of the aggregate are present [see also 4.2.5].

Table 5. Annotation of genes in cluster 4

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
1	Metabolism	DDB0230052	***serA***, 3-phosphoglycerate dehydrogenase	0.92	0.68	0.54	0.41	0.47	0.48	0.58
		DDB0230072	serine hydroxymethyltransferase	0.96	0.74	0.61	0.45	0.47	0.47	0.49
		DDB0191165	***argE***, acetylornithine deacetylase, ornithine biosynthesis	1.00	0.89	0.66	0.63	0.56	0.75	0.97
		DDB0231448	***gabT***, 4-aminobutyrate transaminase, glutamate degradation	0.92	0.92	0.91	0.63	0.67	0.76	0.85
		DDB0231438	glutamate dehydrogenase [NAD(P)+]	0.97	0.89	0.70	0.59	0.55	0.52	0.58
		DDB0218170	***glnB***, glutamate-ammonia ligase	0.95	0.91	0.79	0.64	0.60	0.68	0.80

Results

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
1	Metabolism	DDB0206512	NAD+ dependent glutamate dehydrogenase	0.95	0.75	0.50	0.35	0.38	0.40	0.49
		DDB0231138	kynurenine-oxoglutarate transaminase, glutamate related	0.97	0.88	0.70	0.58	0.57	0.74	0.89
		DDB0230092	***aatA***, aspartate aminotransferase	0.95	0.76	0.61	0.59	0.54	0.68	0.75
		DDB0230190	***bkdA***, branched-chain alpha-keto acid dehydrogenase E1 alpha chain	0.98	0.79	0.60	0.59	0.63	0.84	0.92
		DDB0187236	Proline iminopeptidase.	0.99	0.96	0.74	0.57	0.67	0.69	0.79
		DDB0204020	Ornithine decarboxylase	1.00	0.92	0.76	0.63	0.74	0.82	0.85
		DDB0186679	Probable deoxyhypusine synthase	0.95	0.98	0.86	0.63	0.69	0.88	0.98
		DDB0230070	S-adenosylmethionine synthetase	1.01	0.87	0.58	0.46	0.35	0.37	0.45
		DDB0230137	methylenetetrahydrofolate reductase	0.92	0.87	0.74	0.57	0.55	0.81	0.87
		DDB0188183	pantoate-beta-alanine ligase, pantothenate biosynthesis, alanine related	0.96	0.67	0.84	0.53	0.54	0.42	0.41
		DDB0191167	***spsA***, spermidine synthase from arginine	0.97	1.16	0.92	0.84	0.78	0.72	0.65
		DDB0201565	***purA***, adenylosuccinate synthetase	1.00	0.87	0.73	0.64	0.70	0.76	0.84
		DDB0230095	***purH***, AICAR transformylase / IMP cyclohydrolase	0.99	0.84	0.65	0.52	0.44	0.40	0.56
		DDB0230088	***purC/E***, phosphoribosylaminoimidazole carboxylase	0.93	0.80	0.54	0.44	0.41	0.50	0.70
		DDB0230084	***purD***, phosphoribosylamine-glycine ligase	1.01	0.88	0.59	0.46	0.49	0.50	0.63
		DDB0230083	***purF***, phosphoribosylpyrophosphate amidotransferase	0.96	0.89	0.89	0.67	0.56	0.49	0.67
		DDB0214905	***thyA***, thymidylate synthase (FAD)	1.01	1.01	0.82	0.70	0.60	0.53	0.63
		DDB0191436	***thyB***, thymidine kinase	1.00	0.85	0.71	0.64	0.52	0.56	0.63
		DDB0214958	***pyr56***, bifunctional UMP-synthetase	1.02	1.01	0.87	0.72	0.47	0.36	0.40
		DDB0201646	***pyr1-3***, carbamoyl-phosphate synthase (glutamine-hydrolysing)	1.02	1.10	0.85	0.53	0.50	0.40	0.51
		DDB0230162	CTP synthase	1.00	0.98	0.88	0.59	0.57	0.57	0.58
		DDB0215334	***guaA***, GMP synthetase	0.93	1.06	0.82	0.60	0.53	0.52	0.72
		DDB0230098	***guaB***, IMP dehydrogenase, inosine-5'-monophosphate dehydrogenase	0.93	0.87	0.65	0.38	0.39	0.49	0.86
		DDB0185217	***pyr4***, dihydroorotate oxidase	1.00	0.91	0.76	0.67	0.53	0.53	0.72
		DDB0191172	***pyd2***, dihydropyrimidinase	0.96	0.90	0.81	0.64	0.60	0.68	0.85
		DDB0231475	aldehyde dehydrogenase	0.99	1.12	1.05	0.98	1.03	0.81	0.66
		DDB0231480	aldehyde dehydrogenase	1.02	0.81	0.64	0.70	0.71	0.83	0.74
		DDB0215363	***alrA***, aldehyde reductase	1.02	0.81	0.55	0.51	0.59	0.42	0.39
		DDB0231286	***alrE***, aldo-keto reductase	0.99	0.69	0.56	0.51	0.52	0.46	0.41
		DDB0204808	AhpC/TSA family protein, phospholipid catabolic process	0.97	0.89	0.74	0.65	0.60	0.67	0.83
		DDB0169506	***gtr2***, alpha amylase domain-containing protein, starch synthase-like protein, might be involved in glycogen biosynthesis, GlycosylTRansferase	1.01	1.01	0.86	0.55	0.57	0.58	0.72
		DDB0231456	***gnt3***, alpha-1,3-mannosyl-glycoprotein beta-1,2-N-acetylglucosaminyltransferase, mycothiol biosynthesis	1.06	1.03	0.76	0.79	0.62	0.71	0.77
		DDB0219355	***maoA***, amine oxidase (flavin-containing)	0.96	0.73	0.53	0.46	0.49	0.69	0.86
		DDB0188339	putative amino oxidase	0.97	0.91	0.68	0.60	0.63	0.65	0.73
		DDB0183806	putative sarcosine oxidase and L-amino acid oxidase	1.02	1.03	0.76	0.59	0.66	0.75	0.99
		DDB0231333	***asnS1***, asparagine-tRNA ligase	0.94	0.88	0.62	0.56	0.57	0.64	0.72
		DDB0231311	***maspS***, aspartyl-tRNA synthetase	0.95	0.95	0.77	0.63	0.67	0.69	0.70
		DDB0191481	***sapA***, saposin A, sphingolipid metabolic process	1.01	1.01	0.73	0.47	0.43	0.71	1.06
		DDB0187073	saposin B domain-containing protein	0.90	1.02	0.80	0.63	0.50	0.60	0.84
		DDB0185956	saposin B domain-containing protein	1.06	0.96	0.87	0.76	0.62	0.68	0.88
		DDB0231429	putative glutathione transferase	0.94	0.67	0.54	0.51	0.56	0.45	0.40
		DDB0231434	putative glutathione transferase	1.01	1.02	1.01	0.83	0.84	0.64	0.64
		DDB0217453	Similar to Xenopus laevis (African clawed frog). glutathione s-transferase	0.96	0.78	0.55	0.48	0.62	0.44	0.39
		DDB0230068	beta-ketoacyl synthase family protein, Lipid biosynthesis	0.94	0.97	0.94	0.56	0.45	0.35	0.41
		DDB0188248	acyltransferase activity	0.98	1.07	0.91	0.73	0.66	0.77	0.79
		DDB0191456	***fadA***, delta 5 fatty acid desaturase	0.97	0.85	0.66	0.60	0.57	0.77	0.88
		DDB0217332	Similar to Mortierella alpina. stearoyl-CoA desaturase	0.93	0.67	0.48	0.37	0.36	0.42	0.52
		DDB0191146	***eapA***, alkyl-dihydroxyacetonephosphate synthase	0.93	0.74	0.61	0.46	0.52	0.61	0.64
		DDB0215017	***fps***, farnesyl diphosphate synthase	0.98	0.86	0.65	0.58	0.60	0.64	0.74
		DDB0204468	***pssA***, phosphatidylserine synthase	1.05	1.06	1.01	0.96	0.85	0.60	0.59
		DDB0231376	***fdfT***, farnesyl-diphosphate farnesyltransferase	0.99	0.80	0.66	0.51	0.67	0.74	0.83
		DDB0188166	Endosperm C-24 sterol methyltransferase.	0.92	0.66	0.31	0.16	0.18	0.23	0.47
		DDB0190993	ergosterol biosynthesis	0.98	0.88	0.74	0.65	0.61	0.60	0.66

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
1	Metabolism	DDB0215357	***hmgB***, hydroxymethylglutaryl CoA reductase	0.96	0.72	0.63	0.57	0.59	0.55	0.66
		DDB0189754	esterase/lipase/thioesterase domain	0.98	0.81	0.77	0.60	0.66	0.62	0.56
		DDB0231380	***plbE***, phospholipase B-like protein	1.05	0.95	0.80	0.68	0.62	0.65	0.74
		DDB0202574	Sterol 24-C-methyltransferase	0.99	0.77	0.55	0.36	0.36	0.30	0.40
		DDB0188084	putative acyl-CoA oxidase	0.98	0.82	0.85	0.66	0.72	0.87	0.93
		DDB0231414	***hemF***, coproporphyrinogen III oxidase	1.07	0.64	0.63	0.88	0.91	0.79	0.73
		DDB0185491	***gcdh***, glutaryl-CoA dehydrogenase	0.94	0.80	0.67	0.63	0.65	0.76	0.84
		DDB0217316	Nucleoside diphosphate kinase	0.93	0.99	1.00	0.78	0.61	0.73	0.84
		DDB0188870	pantothenate kinase	0.96	0.80	0.71	0.71	0.66	0.58	0.58
		DDB0219237	***agl***, glycogen debranching enzyme	0.95	0.92	0.75	0.65	0.71	0.87	1.00
		DDB0218661	enoyl-CoA hydratase	0.94	0.87	0.64	0.61	0.66	0.77	0.88
		DDB0185740	esterase/lipase/thioesterase domain-containing protein	1.01	0.90	0.85	0.59	0.57	0.44	0.38
		DDB0231108	***pckA***, phosphoenolpyruvate carboxykinase	1.00	0.67	0.54	0.56	0.61	0.66	0.65
		DDB0185087	***gpdA***, glyceraldehyde-3-phosphate dehydrogenase	0.93	0.86	0.68	0.69	0.65	0.77	0.75
		DDB0231355	***enoA***, phosphopyruvate hydratase	0.94	0.81	0.74	0.67	0.61	0.84	0.88
		DDB0231387	***fba***, fructose-bisphosphate aldolase, glycolysis	1.03	0.79	0.61	0.54	0.57	0.72	0.86
		DDB0216232	***lpd***, dihydrolipoamide:NAD oxidoreductase	0.94	0.85	0.65	0.58	0.75	0.76	0.91
		DDB0217233	Similar to Oryza sativa glucose-6-phosphate dehydrogenase (G6PD)	1.07	0.70	0.60	0.56	0.47	0.52	0.57
		DDB0217422	Similar to Listeria monocytogenes. Tkt protein.	0.98	0.79	0.69	0.61	0.75	0.76	0.80
		DDB0215011	***gnd***, 6-phosphogluconate dehydrogenase (decarboxylating)	0.92	0.90	0.89	0.73	0.72	0.58	0.68
		DDB0205389	***acly***, ATP citrate synthase	0.92	0.83	0.71	0.64	0.66	0.56	0.48
		DDB0231288	***idhA***, isocitrate dehydrogenase (NAD+)	0.96	0.90	0.66	0.65	0.64	0.74	0.69
		DDB0205386	putative ATP citrate lyase	1.04	0.78	0.51	0.41	0.40	0.38	0.31
		DDB0220638	citrate synthase, mitochondrial	0.94	0.75	0.56	0.51	0.55	0.80	0.75
		DDB0185935	***ppa1***, inorganic pyrophosphatase	0.94	0.90	0.72	0.68	0.66	0.69	0.73
		DDB0187875	1,4-beta-D-xylan xylohydrolase	0.97	0.94	0.71	0.63	0.58	0.64	0.79
		DDB0232952	***ptsA***, 6-pyruvoyltetrahydropterin synthase	0.98	0.73	0.61	0.60	0.58	0.51	0.53
		DDB0191399	***gchA***, GTP cyclohydrolase I	0.97	0.87	0.83	0.56	0.57	0.52	0.47
		DDB0230118	***thfA***, methylenetetrahydrofolate dehydrogenase	0.98	0.99	0.84	0.71	0.55	0.48	0.55
		DDB0169112	NAD-dependent epimerase/dehydratase family protein	0.97	0.85	0.77	0.68	0.61	0.50	0.57
		DDB0188270	Similar to Lactococcus lactis oxidoreductase	1.00	0.89	0.69	0.58	0.59	0.62	0.76
2	Energy	DDB0191419	***vatC***, H(+)-transporting ATPase	1.02	0.93	0.67	0.65	0.58	0.73	1.00
		DDB0167892	vacuolar ATP synthase subunit D	0.97	0.92	0.62	0.56	0.48	0.78	1.07
		DDB0185207	***vatB***, vacuolar H+ ATPase B subunit	0.96	1.01	0.67	0.56	0.52	0.73	1.11
		DDB0201563	***vatA***, vacuolar H+-ATPase A subunit	0.99	0.87	0.63	0.60	0.51	0.62	0.88
		DDB0185070	***vatE***, vacuolar H+-ATPase E subunit	0.94	0.90	0.64	0.52	0.50	0.63	0.91
		DDB0216933	Similar to H+-transporting ATPase.	0.99	0.88	0.61	0.61	0.54	0.67	1.02
		DDB0217242	Similar to V-ATPase d subunit	0.99	0.92	0.74	0.68	0.65	0.66	1.04
		DDB0185431	ATP synthesis coupled proton transport	0.98	0.79	0.65	0.57	0.63	0.88	1.00
		DDB0184316	***atp5C1***, ATP synthase F1 subunit alpha.	0.99	0.84	0.71	0.63	0.67	0.85	0.93
		DDB0217763	***atp5e***, ATP synthase epsilon chain, mitochondrial	1.03	0.79	0.57	0.61	0.67	0.78	0.81
		DDB0216257	***cytC***, cytochrome c	0.91	0.96	0.91	0.68	0.64	0.72	0.71
		DDB0214995	***cxdA***, cytochrome c oxidase subunit IV	1.02	0.77	0.62	0.67	0.59	0.75	0.83
		DDB0191104	***cxeA***, cytochrome c oxidase subunit V	0.93	0.86	0.77	0.62	0.69	0.76	0.87
		DDB0218167	***CYP51***, cytochrome P450 family protein	0.95	0.71	0.51	0.36	0.36	0.36	0.51
		DDB0202357	***CYP524A1***, cytochrome P450 family protein	0.95	0.84	0.71	0.70	0.60	0.42	0.53
		DDB0201618	***nad11***, NADH dehydrogenase subunit 11	1.01	1.29	1.21	1.08	0.79	0.73	0.55
		DDB0218707	NADH-cytochrome B5 reductase	0.96	0.91	0.70	0.61	0.64	0.85	0.95
		DDB0184465	Similar to cytochrome c-1	0.94	0.83	0.66	0.54	0.52	0.69	0.76
3	Transcription	DDB0216420	***hmg2***, HMG1/2 (high mobility group) box-containing protein, regulation of transcription	0.93	0.75	0.54	0.51	0.53	0.63	0.72
		DDB0216404	myb domain-containing protein	0.96	0.97	0.62	0.61	0.67	0.66	0.78
		DDB0216409	paired amphipathic helix (PAH) containing protein	1.03	0.68	0.49	0.53	0.55	0.61	0.68
		DDB0233426	putative GATA-binding transcription factor, GATA Zn finger-containing protein	1.01	0.90	0.69	0.74	0.63	0.68	0.74
		DDB0216292	***rpa1***, RNA polymerase I, largest subunit	1.04	1.19	0.93	0.73	0.70	0.52	0.52
		DDB0204655	Lupus La protein, plays a role in the transcription of RNA polymerase III	1.00	1.06	0.85	0.62	0.59	0.72	0.77
		DDB0191270	***trfA***, homologous to yeast Ssn6, a transcription factor	1.07	0.94	0.70	0.71	0.61	0.74	0.76

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
4	Translation	DDB0231059	***rps19***, 40S ribosomal protein S19	0.87	1.06	0.94	0.76	0.61	0.72	0.78
		DDB0231066	***rps29***, 40S ribosomal protein S29	1.05	1.13	0.93	0.79	0.64	0.81	0.78
		DDB0230022	***rps5***, 40S ribosomal protein S5	0.95	1.16	0.84	0.74	0.64	1.02	0.82
		DDB0230023	***rps6***, 40S ribosomal protein S6	0.92	0.96	0.70	0.70	0.59	0.70	0.79
		DDB0230024	***rps7***, 40S ribosomal protein S7	0.95	1.06	0.76	0.84	0.57	0.61	0.71
		DDB0231192	***rpl13a***, S60 ribosomal protein L13a	0.79	1.13	0.85	0.90	0.65	0.68	0.84
		DDB0229959	***rpl17***, S60 ribosomal protein L17	0.91	1.00	0.79	0.73	0.58	0.65	0.70
		DDB0229960	***rpl18***, S60 ribosomal protein L18	0.90	0.82	0.82	0.72	0.60	0.67	0.72
		DDB0229962	***rpl21***, S60 ribosomal protein L21	0.92	0.97	0.84	0.77	0.63	0.67	0.78
		DDB0230149	***rpl23a***, S60 ribosomal protein L23a	0.81	1.01	0.70	0.60	0.65	0.60	0.69
		DDB0230153	***rpl27***, S60 ribosomal protein L27	0.97	0.89	0.85	0.72	0.60	0.72	0.72
		DDB0201638	***rpl27a***, S60 ribosomal protein L27a	0.86	0.99	0.81	0.63	0.65	0.62	0.68
		DDB0230155	***rpl3***S60 ribosomal protein L30	0.99	0.99	0.81	0.70	0.65	0.71	0.74
		DDB0231150	***rpl32***, S60 ribosomal protein L32	0.93	1.00	0.88	0.70	0.61	0.68	0.72
		DDB0231151	***rpl34***, S60 ribosomal protein L34	0.91	0.94	0.84	0.69	0.64	0.72	0.74
		DDB0185073	***rpl7***, S60 ribosomal protein L7	0.89	0.97	0.81	0.71	0.65	0.74	0.77
		DDB0231241	***rpl4***, 60S ribosomal protein L4	0.86	0.98	0.91	0.86	0.59	0.71	0.77
		DDB0231338	***rpl6***, 60S ribosomal protein L6	0.97	0.95	0.80	0.72	0.61	0.63	0.69
		DDB0191528	***rps9***, ribosomal protein 1024, 40S ribosomal protein S9	0.91	1.06	0.80	0.76	0.60	0.76	0.87
		DDB0168982	***rlp24***, ribosomal protein L24-like protein	0.95	0.97	0.76	0.79	0.76	0.70	0.66
		DDB0205581	Ribsomal protein-like (At5g09770).	0.89	0.77	0.64	0.61	0.70	0.87	0.82
		DDB0237471	***17S rRNA-2***, 17S ribosomal RNA	0.90	1.15	0.98	0.89	0.94	0.82	0.60
		DDB0231374	translation initiation factor eIF-2B alpha subunit	0.99	1.00	1.04	0.97	0.93	0.72	0.65
		DDB0214990	***eRF3***, eukaryotic release factor 3	0.93	0.89	0.71	0.63	0.68	0.85	1.01
		DDB0204044	***eIF3s3***, eukaryotic translation initiation factor 3 (eIF3) subunit 3	0.94	0.86	0.68	0.66	0.67	0.79	0.80
		DDB0216584	***eIF4e***, eukaryotic translation initiation factor 4E	1.00	0.66	0.41	0.49	0.58	0.74	0.80
		DDB0191174	***efa1B***, elongation factor 1b	1.08	0.92	0.68	0.70	0.60	0.77	0.85
		DDB0191363	***efbA***, elongation factor 2	0.93	0.94	0.76	0.65	0.64	0.68	0.76
		DDB0219464	***tufM***, elongation factor Tu domain-containing protein	0.98	0.83	0.53	0.51	0.46	0.43	0.47
		DDB0205289	***nhp2l1***, non-histone chromosome protein 2-like 1	0.91	0.99	0.65	0.48	0.45	0.44	0.45
5	Protein destination: Protein folding and stabilization / proteolysis / protein targeting, sorting and translocation	DDB0185614	***ppiD***, cyclophilin-type peptidylprolyl cis-trans isomerase (PPIase)	1.01	0.87	0.70	0.63	0.70	0.85	0.88
		DDB0191163	***hspD***, heat shock cognate protein	0.97	0.85	0.69	0.60	0.60	0.67	0.88
		DDB0219929	***hspA***, chaperonin 60	0.99	0.77	0.55	0.63	0.63	0.80	0.85
		DDB0205559	putative nascent polypeptide-associated complex alpha subunit	0.99	1.01	0.93	0.87	0.80	0.60	0.64
		DDB0215016	***ddj1***, heat shock protein	0.93	0.60	0.44	0.38	0.49	0.69	0.78
		DDB0215005	***cprG***, cysteine proteinase	0.87	1.16	0.75	0.54	0.55	0.80	1.09
		DDB0219654	Cysteine proteinase 1 precursor	1.04	0.90	0.67	0.61	0.55	0.67	0.96
		DDB0167572	peptidase C19 family protein	1.01	0.99	0.71	0.65	0.46	0.59	0.70
		DDB0206429	peptidase C1A family protein	0.94	1.00	0.75	0.64	0.67	0.81	0.96
		DDB0189005	Putative ubiquitin-conjugating enzyme	0.96	0.92	0.82	0.76	0.66	0.73	0.73
		DDB0186598	Similar to cell division cycle 20 homolog (S. cerevisiae)	0.98	0.86	0.65	0.63	0.58	0.60	0.70
		DDB0188097	***mppB***, mitochondrial processing peptidase beta subunit	1.02	0.82	0.66	0.70	0.72	0.74	0.83
7	Transport	DDB0191229	***abcG2***, ABC transporter G family protein	0.99	1.11	1.70	1.16	0.64	0.47	0.54
		DDB0185017	***amtA***, ammonium transporter	1.00	0.93	0.65	0.49	0.49	0.71	1.19
		DDB0185213	***porA***, porin	0.90	0.75	0.59	0.43	0.46	0.54	0.65
		DDB0202615	***nramp1***, solute carrier family 11 member 1, ion transport	1.00	0.80	0.66	0.57	0.63	0.86	0.98
		DDB0201558	***ancA***, ADP/ATP translocase	0.95	0.86	0.64	0.45	0.45	0.47	0.55
		DDB0192069	***slc25a3***, phosphate carrier protein, mitochondrial substrate carrier family protein	1.01	0.85	0.65	0.52	0.46	0.48	0.57
		DDB0229992	***mcfZ***, mitochondrial substrate carrier family protein, succinate:fumarate antiporter activity	0.94	0.78	0.69	0.54	0.51	0.47	0.47
		DDB0217211	Similar to Mus musculus (Mouse) importin alpha-1 subunit	0.93	1.06	0.82	0.70	0.61	0.51	0.64
		DDB0191099	***fhbA***, flavohemoglobin	0.98	0.59	0.80	0.52	0.81	0.52	0.41
		DDB0168979	NAPC PROTEIN, Member of the Major Facilitator Superfamily (MFS)	0.96	0.43	0.31	0.27	0.41	0.26	0.28
8	Cell proliferation	DDB0187545	***PCNA***, proliferating cell nuclear antigen, DNA elongation	0.99	1.06	0.79	0.70	0.63	0.69	0.69

	Category	DDB ID	Annotation	Differential regulation						
				T0	T15	T30	T45	T60	T90	T120
9	Movement / cytoskeletal	DDB0215369	***coaA***, actin binding protein	0.96	0.75	0.58	0.53	0.53	0.90	0.92
		DDB0219923	***comA***, actin binding protein	0.99	0.84	0.75	0.71	0.59	0.67	0.77
		DDB0215000	***forB***, actin binding protein	1.08	0.87	0.70	0.68	0.64	0.65	0.66
		DDB0187112	putative actin binding protein	1.00	0.99	0.89	0.87	0.71	0.65	0.62
		DDB0215336	***hatB***, hisactophilin II	0.95	0.72	0.46	0.40	0.41	0.70	0.95
		DDB0191178	***proA***, profilin I	1.00	0.79	0.59	0.59	0.60	0.78	0.87
		DDB0191380	***tubA***, alpha tubulin	1.02	0.87	0.63	0.59	0.58	0.63	0.79
		DDB0191169	***tubB***, beta tubulin	0.95	0.80	0.62	0.59	0.65	0.81	1.00
		DDB0204271	Tubulin alpha-1B chain	0.92	0.87	0.70	0.66	0.66	0.83	0.93
		DDB0215380	***ponA***, ponticulin	1.03	0.91	0.82	0.82	0.67	0.60	0.67
		DDB0201555	***kif12***, kinesin family member 12	1.00	1.02	0.87	0.76	0.66	0.75	0.85
10	Stress response	DDB0185047	***hspE***, heat shock protein	1.00	0.91	0.88	0.78	0.75	0.64	0.65
		DDB0217225	Heat shock protein 70	0.98	0.85	0.78	0.73	0.70	0.70	0.64
		DDB0185048	***hspC***, heat shock protein	1.07	0.72	0.54	0.64	0.73	0.71	0.71
		DDB0167206	***hspK***, heat shock protein Hsp20 domain-containing protein	1.00	0.81	0.86	0.70	0.70	0.55	0.52
		DDB0167089	heat shock protein Hsp70 family protein	1.01	0.81	0.68	0.63	0.57	0.73	0.69
		DDB0191276	***hspH***, heat shock protein Hsp70 family protein	1.01	0.55	0.49	0.50	0.55	0.62	0.65
		DDB0215366	***mhsp7***, mitochondrial Hsp70 precursor	1.03	0.72	0.46	0.54	0.52	0.45	0.40
		DDB0191384	***crtA***, calreticulin	0.93	0.71	0.57	0.52	0.58	0.83	0.91
11	Signal transduction	DDB0191507	***rab7A***, Rab GTPase	1.06	0.91	0.81	0.76	0.60	0.74	0.80
		DDB0230034	***rabR***, Rab GTPase	1.01	0.50	0.64	0.50	0.58	0.48	0.49
		DDB0187464	Ran-specific GTPase-activating protein (Ran binding protein 1)	0.97	0.70	0.51	0.52	0.56	0.68	0.87
		DDB0183840	***grlE***, G-protein-coupled receptor (GPCR) family protein	1.02	0.96	0.80	0.65	0.74	0.61	0.64
		DDB0219974	***pdsA***, cAMP phosphodiesterase	1.03	0.76	0.65	0.45	0.46	0.57	0.73
		DDB0185036	***trap1***, TNF receptor-associated protein	0.94	0.76	0.64	0.61	0.61	0.60	0.63
		DDB0185089	***ctnA***, component of the counting factor (CF) complex	0.99	0.96	0.83	0.64	0.60	0.63	0.87
		DDB0191525	***smlA***, regulate conting factor, glucose homeostasis	0.96	0.92	0.54	0.52	0.45	0.56	0.80
		DDB0191095	***cmfB***, putative CMF receptor CMFR1	0.94	0.84	0.55	0.43	0.38	0.34	0.43
		DDB0229453	***ndrD***, putative protein serine/threonine kinase	0.85	0.89	0.68	0.54	0.56	0.61	0.82
		DDB0185028	***cdk1***, p34-cdc2 protein, cyclin-dependent kinase	0.92	0.80	0.55	0.40	0.51	0.65	0.82

See table 3 for legend.

3.2.5 Differential regulation of major metabolic pathway genes

Since the accumulation of osmolytes is a general and important strategy for the adaptation of all organisms to hypertonicity, we checked the differential expression of genes encoding proteins in major metabolic pathways, including carbohydrate, amino acid and nucleotide metabolism. Data were collected from tables 2-5.

3.2.5.1 Carbohydrate metabolism

In liquid culture of vegetative *Dictyostelium* cell, glucose is the main carbon source and glycogen concentration is relatively low, but during development glycogen is synthesized and accumulated (Rutherford, 1976). We checked the genes involved in the breakdown of glycogen, in glycolysis, in the TCA cycle and the synthesis of intermediate components (Figure 26). Obviously glycolysis and the TCA cycle are down-regulated. Glucose seems to be generated from glycogen as well as from other carbon sources, and is probably then used for the synthesis of the compatible osmolyte trehalose.

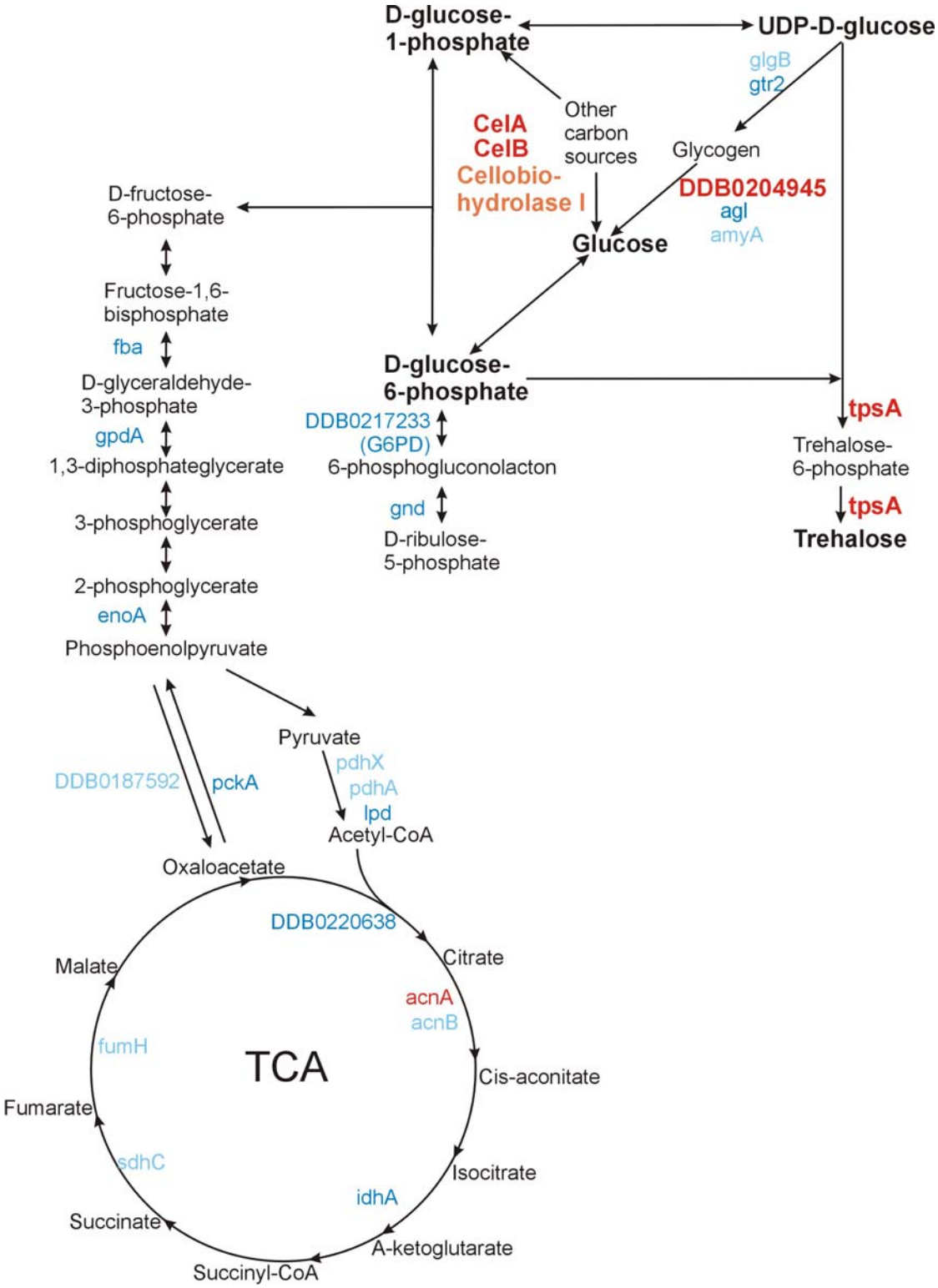

Figure 26. Genes involved in the carbohydrate metabolism pathway. Differentially regulated genes encoding the corresponding enzymes for the catalytic reactions are indicated. Blue indicates down-regulated and red or orange indicates up-regulated genes. Genes from cluster 1 are orange labelled, cluster 2 light blue, cluster 3 red and cluster 4 blue.

Correspondingly many genes encoding components of the aerobic respiration chain were down-regulated (Figure 27).

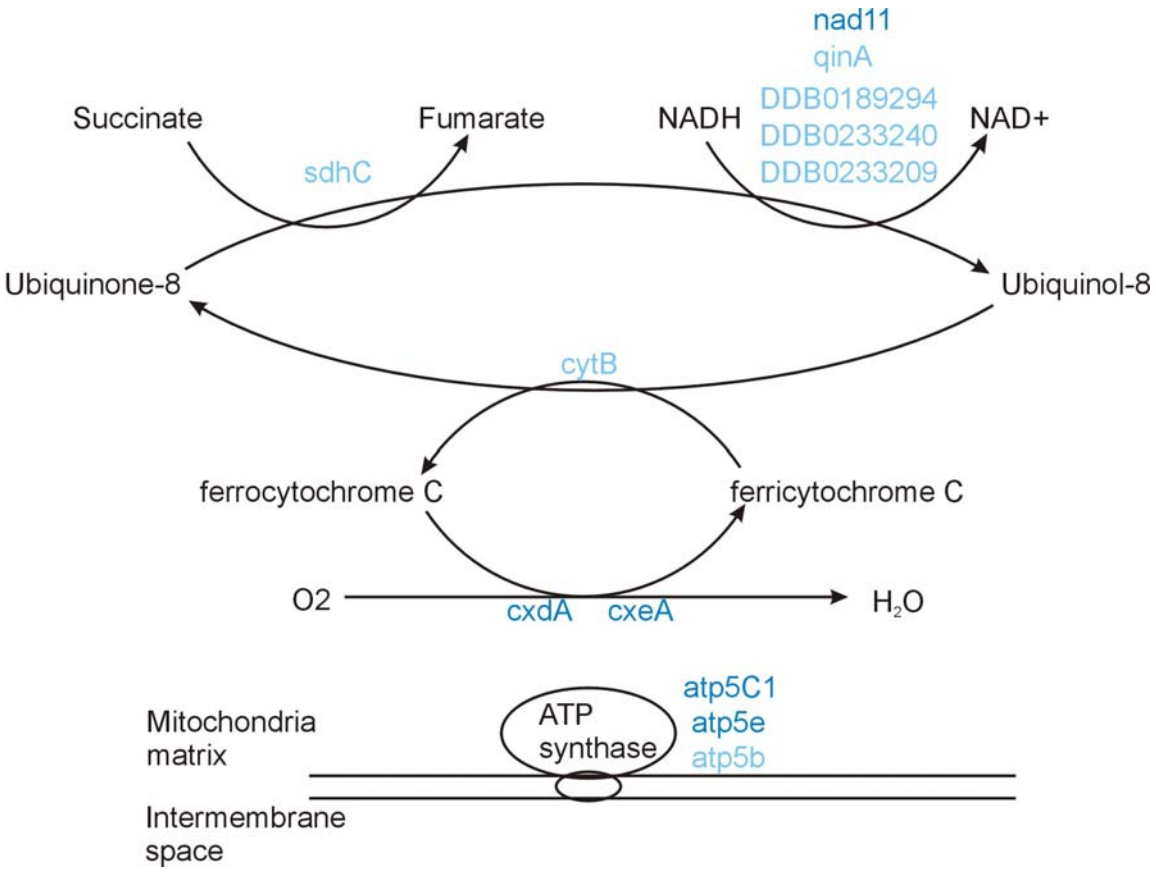

Figure 27. Genes involved in the aerobic respiration chain. See figure 26 for figure legend.

3.2.5.2 Amino acid metabolism

Amino acids are widely used as osmolytes in cell volume regulation by prokaryotes, microbial eukaryotes and metazoans alike (Cronkite et al., 1993; D. L. Cronkite, 1989; Leon Goldstein, 1994). The accumulation of amino acids is specific and selective: in *E.coli*, the main accumulated amino acids are glutamate, glycine and proline (Wood, 2006); in *Dictyostelium*, glycine, alanine and proline are the major amino acids accumulated in response to osmotic stress (Steck et al., 1997). It also has been shown that hyperosmotic stress induces ubiquitination of cellular proteins, which indicates protein degradation (Zischka et al., 1999). It is reasonable to assume that protein degradation leads to free amino acids in the cytoplasm and that through conversion between different amino acids some of them get enriched. Thus we focused on the

genes involved in the metabolism of these amino acids (Figure 28). It is apparent that the metabolism of glutamate is down-regulated. This strategy is similar to that of *E.coli*, which accumulates glutamate though suppression of glutamate catabolism (Wood, 2006). The gene encoding the key enzyme (pyrroline-5-carboxylate reductase) for the synthesis of proline is highly upregulated. Genes encoding aminotransferases were also found to be differentially regulated. For example, *DDB0188646* is upregulated, which encodes alanine-glyoxylate aminotransferase and serine-pyruvate transaminase, was up-regulated and thus could affect the conversion to alanine, glycine and serine. The down-regulated *DDB0230072* encodes the serine hydroxymethyltransferase, which could convert serine to glycine or vice versa. Since the metabolic machinery for the synthesis or breakdown of glutamate, proline, alanine, glycine and serine is changed, we assume that *Dictyostelium* accumulates a mixture of amino acids to achieve sufficient intracellular osmolarity to counteract the extracellular osmolarity. This is in agreement with the results of Steck et al., however, further biochemical evidence needs to be obtained to prove this hypothesis (Steck et al., 1997).

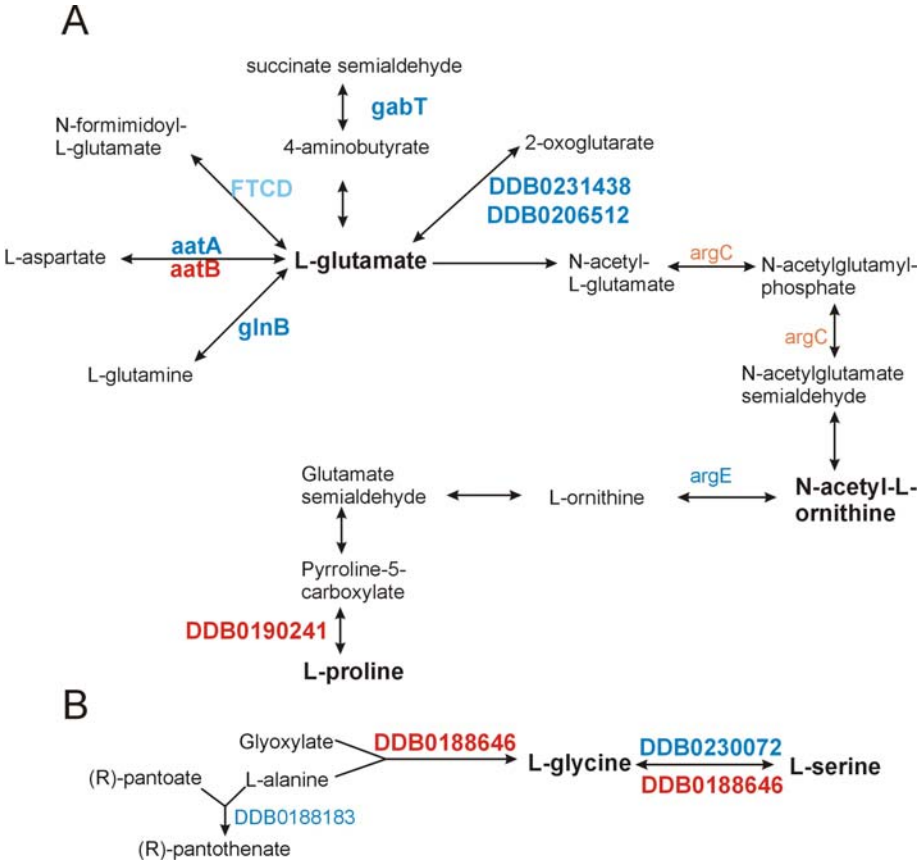

Figure 28. Genes involved in amino acids metabolism. See figure 26 for figure legend.

3.2.5.3 Purine and pyrimidine metabolism

Most genes involved in the purine and pyrimidine metabolic pathways were significantly down-regulated, except those in the pathway from AMP to glyoxylate and urea, which could act as osmolyte (Figure 29).

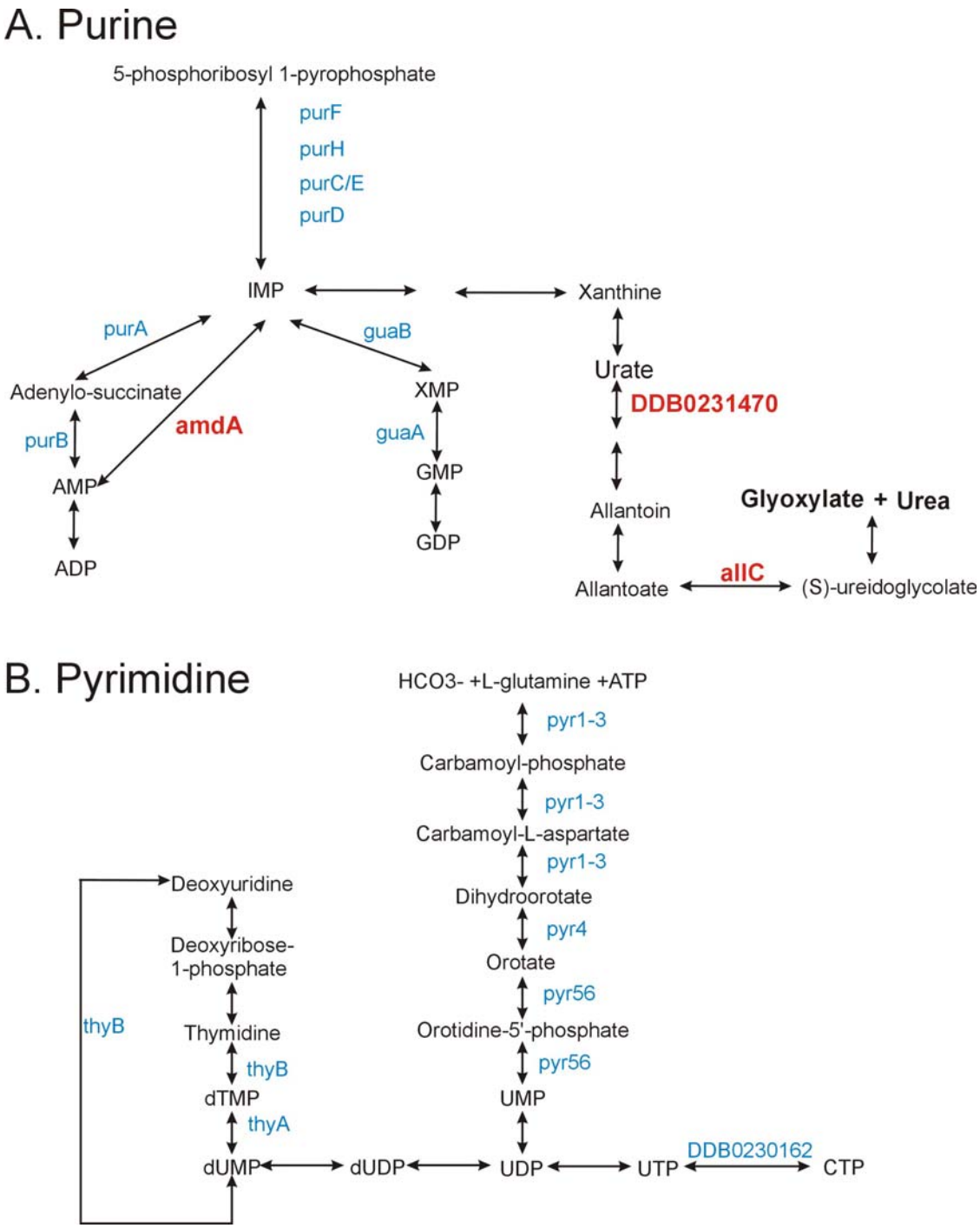

Figure 29. Genes involved in purine and pyrimidine metabolism. See figure 26 for figure legend.

3.3 Characterization of the early transcriptional response genes

The early transcriptional response of the cells to hyperosmotic stress is particularly interesting because these genes are apparently primary targets of the signaling cascade(s) that redirect(s) the transcriptional program of the cells. Furthermore, individual components of the signaling cascade(s) might be differentially regulated and it is also feasible that the responsible transcription factor(s) autoregulate(s) its/their own expression via a positive or negative feed back loop. Therefore, we focused on the early differentially regulated genes of the time course experiment (Table 6). The analysis revealed several interesting up-regulated genes encoding SrfA induced genes (see below), STATc (see below), FcpA (a putative C-terminal phosphatase that could play a regulatory role in the response to hyperosmolarity) and RasGapA (an IQGAP-related protein involved in the completion of cytokinesis). In addition, genes for several transporters, two Cyclin_N domain containing proteins, RabR and the eukaryotic translation initiation factor 4E, which has a significant function in the initiation of eukaryotic protein synthesis, were reported. Besides, two genes encoding transmembrane proteins are reported, one of which is cAMP receptor 1.

Table 6. Selection of early differentially expressed genes with unambiguous annotation

DDB ID	Annotation	Differential regulation						
		T0	T15	T30	T45	T60	T90	T120
DDB0191392	*sig*G: *srf*A induced gene G	1.05	2.40	4.87	6.68	5.58	1.91	0.91
DDB0191111	*sigJ*: *srf*A induced gene J	0.99	2.29	5.64	6.47	6.36	5.63	5.24
DDB0218006	Homologue of human cyclin fold protein 1	1.00	2.04	3.40	2.57	1.99	0.93	0.82
DDB0201666	AbcB1: ABC transporter B family protein	1.10	1.98	2.92	3.32	2.80	1.63	1.05
DDB0229894	FcpA: putative CTD (C-terminal domain) phosphatase	1.00	1.85	2.98	3.17	3.28	2.31	1.45
DDB0189650	Na^+/K^+ ATPase; Na^+/K^+-transporting ATPase alpha chain 2	1.03	1.71	3.24	3.09	3.15	1.36	0.87
DDB0206314	Putative transmembrane protein; 6-TM domains	1.00	1.67	2.55	2.63	2.71	1.92	1.19
DDB0169197	Protein contains Cyclin_N domain	1.03	1.65	2.17	1.37	1.25	0.98	1.01
DDB0191293	GapA: RasGTPase-activating protein	0.91	1.58	2.31	1.74	1.44	0.76	0.65
DDB0215378	STATc: STAT family protein	0.98	1.57	3.05	3.18	3.41	2.13	1.11
DDB0191122	*celB*, cellulose binding protein	0.93	1.96	3.83	5.59	4.08	2.11	1.39
DDB0190241	pyrroline-5-carboxylate reductase	0.94	1.72	2.15	2.56	2.59	2.42	2.02
DDB0203727	antioxidant enzyme	0.88	2.29	3.80	5.52	6.09	5.85	3.36
DDB0217181	Cyclic AMP receptor 1	0.95	1.70	1.80	1.08	0.90	0.78	0.84
DDB0216584	Eucaryotic translation initiation factor 4E	1.00	0.66	0.41	0.49	0.58	0.74	0.80
DDB0230034	*rabR*: rab GTPase R	1.02	0.50	0.64	0.50	0.58	0.48	0.49
DDB0168979	Member of the Major Facilitator Superfamily (MFS)	0.96	0.43	0.31	0.27	0.41	0.26	0.28

SrfA: Serum Response Factor A; ABC transporter: ATP-Binding Cassette transporter; FcpA: Transcription factor IIF (TFIIF)-associating CTD phosphatase; red: up-regulated; blue: down-regulated.

3.3.1 SrfA is not involved in the transcriptional response to hyperosmotic stress

We found a number of genes 15 minutes after treatment and at later time points that had been shown to be regulated by the MADS box transcription factor SrfA (Escalante et al., 2004a). In addition, *srfA* itself was slightly up-regulated at the time point t_{30} (Table 4), indicating a possible involvement of SrfA in the differential regulation of target genes. Therefore, we tested the role of SrfA in the *Dictyostelium* response to hyperosmotic conditions by comparing the transcriptional profiles of AX2 wild type and *srf*A$^-$ cells. The results showed that, out of 46 genes reported by SAM, only 10 genes were differentially regulated more than 1.5 fold and the fold change values were very low (Table 7). We conclude that the transcription factor SrfA is probably not involved in the transcriptional response to hyperosmotic stress.

Table 7. Osmotic stress experiments with wt and srfA ko cells.

Gene ID	Annotation	Differential regulation
DDB0216989	similar to histidine kinase DhkL	1,632
DDB0231570	***alp***, alkaline phosphatase	1,580
DDB0167826	unknown	1,539
DDB0215343	***lmcB***, vegetative-specific gene repressed at the onset of development	1,527
DDB0219750	unknown	1,524
DDB0167628	unknown	1,515
DDB0167628	unknown	1,508
DDB0219496	unknown	1,507
DDB0191484	***RPLP2***, ribosomal acidic phosphoprotein P2	0,628

RNA was isolated from 3 independent cultures and 6 microarrays were analyzed. Experiment: AX2 wild type cell; control: srfA ko cell.

3.3.2 STATc is a key regulator of the transcriptional response to hyperosmotic stress

A very interesting member of the early differentially regulated genes was STATc (Table 6 and Figure 23). It had already been shown that STATc is activated in cells subjected to different types of stress (Araki et al., 2003) and we reasoned that STATc might be a transcriptional regulator in the *Dictyostelium* response to hyperosmotic conditions. To test this hypothesis we performed microarray experiments with either treated or untreated AX2 wt cells (wt; experiment I), the STATc knock-out mutant (STATc$^-$, experiment III) and a mutant with an isogenic background where the STATc knock-out construct was randomly integrated into the genome (RIC, experiment II) (Table 8).

Table 8. Osmotic stress experiments with wt, STATc ko and RIC cells.

Comparison	Experiment	Control	Independent cultures	Microarrays
I	wt **+**	wt **-**	8	16
II	RIC **+**	STATc ko **+**	3	6
III	STATc ko **+**	STATc ko **-**	3	6

wt: AX2 wild type cells; ko: knock-out; RIC: random integrant cells; **+**: treated; **-**: untreated.

Cells were treated for one hour with 200 mM sorbitol and the lists of differentially regulated genes were compared between the different experiments. There are several possible outcomes: i) If STATc is not involved in the osmostress response we would have expected a complete overlap of the differentially regulated genes in experiments I and III (Fig. 30A). ii) If STATc is the only transcriptional regulator that directly gets activated in sorbitol-treated *Dictyostelium* cells we would have expected no differential regulation in experiment III and a complete overlap of the gene lists in experiments I and II (Fig. 30B). iii) If STATc is only partially responsible for the transcriptional response a more complicated output was expected and this was the case. A set of 117 genes was identified, that was common to all three comparisons and sets of 388, 149 and 87 genes, respectively, that were common to two comparisons (Fig. 30C).

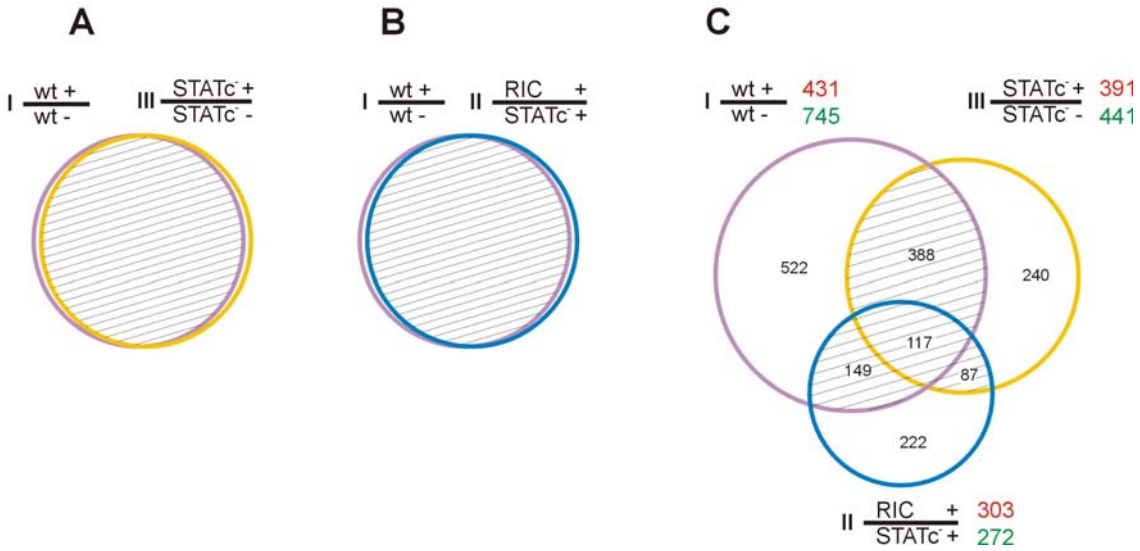

Figure 30. STATc is an important regulator of the transcriptional response to hyperosmotic stress. (A) Expected overlap of differentially regulated genes in experiments I and III if STATc is not involved in the transcriptional regulation. (B) Expected overlap in experiments I and II if STATc is the only transcriptional regulator in response to hypertonicity. (C) Venn diagram of the observed differentially regulated genes from the three comparisons: wt cells treated versus untreated (I), RIC cells treated versus STATc- treated (II) and STATc- treated versus untreated (III). Up-regulated genes are printed in red and down-regulated genes in green. Genes shared between 2 or 3 comparisons (shaded region) were applied to further analysis.

The 149 differentially regulated genes that were common between experiments I and II appear to be solely regulated by activated STATc. In contrast STATc is only partially responsible for the regulation of the 117 and 87 differentially regulated genes, respectively. Interestingly, 82 of the 87 genes common between experiments II and III were oppositely regulated in these experiments resulting in a balanced output in experiment I (data not shown). We conclude that STATc is a major but not the only transcriptional regulator in the *Dictyostelium* response to hyperosmotic conditions.

If we assume two parallel signaling pathways that are activated upon hyperosmotic stress and in addition a STATc pathway independent of osmostress we need to consider three factors that influence the transcriptional output of every gene in our comparisons: i) regulation by the osmostress pathway 1 (OP1), ii) regulation by the osmostress-induced STATc pathway (OSP) and iii) regulation by the STATc pathway (SP) irrespective of osmostress. The transcription of a given gene is either independent of these factors (majority of genes) or activated or repressed. Since we are dealing in the analysis with three different factors and three possible regulations there are 27 cases that need to be considered, some of which result in identical outputs for the three comparisons (Table 9).

Table 9. Possible regulations of target genes by different signaling pathways, pathways involved in the comparisons and expected regulatory output of target genes.

Case	Possible regulatory combinations			Comparison			Expected regulatory output		
	OP1	OSP	SP	WT +/WT -	RIC +/STATc ko +	STATc ko +/STATc ko -	WT +/WT -	RIC +/ STATc ko +	STATc ko +/ STATc ko -
				Pathways involved					
				OP1*OSP*SP/SP	OP1*OSP*SP/OP1	OP1	Expression		
1	↓	↓	↓	OP1*OSP	OSP*SP	OP1	↓	↓	↓
2	↑	↓	↓	OP1*OSP	OSP*SP	OP1	↑/↓/0	↓	↑
3	0	↓	↓	OP1*OSP	OSP*SP	OP1	↓	↓	0
4	↓	↓	↑	OP1*OSP	OSP*SP	OP1	↓	↑/↓/0	↓
5	↑	↓	↑	OP1*OSP	OSP*SP	OP1	↑/↓/0	↑/↓/0	↑
6	0	↓	↑	OP1*OSP	OSP*SP	OP1	↓	↑/↓/0	0
7	↓	↓	0	OP1*OSP	OSP*SP	OP1	↓	↓	↓
8	↑	↓	0	OP1*OSP	OSP*SP	OP1	↑/↓/0	↓	↑
9	0	↓	0	OP1*OSP	OSP*SP	OP1	↓	↓	0
10	↓	↑	↓	OP1*OSP	OSP*SP	OP1	↑/↓/0	↑/↓/0	↓
11	↑	↑	↓	OP1*OSP	OSP*SP	OP1	↑	↑/↓/0	↑
12	0	↑	↓	OP1*OSP	OSP*SP	OP1	↑	↑/↓/0	0
13	↓	↑	↑	OP1*OSP	OSP*SP	OP1	↑/↓/0	↑	↓
14	↑	↑	↑	OP1*OSP	OSP*SP	OP1	↑	↑	↑
15	0	↑	↑	OP1*OSP	OSP*SP	OP1	↑	↑	0
16	↓	↑	0	OP1*OSP	OSP*SP	OP1	↑/↓/0	↑	↓
17	↑	↑	0	OP1*OSP	OSP*SP	OP1	↑	↑	↑
18	0	↑	0	OP1*OSP	OSP*SP	OP1	↑	↑	0
19	↓	0	↓	OP1*OSP	OSP*SP	OP1	↓	↓	↓
20	↑	0	↓	OP1*OSP	OSP*SP	OP1	↑	↓	↑
21	0	0	↓	OP1*OSP	OSP*SP	OP1	0	↓	0
22	↓	0	↑	OP1*OSP	OSP*SP	OP1	↓	↑	↓
23	↑	0	↑	OP1*OSP	OSP*SP	OP1	↑	↑	↑
24	0	0	↑	OP1*OSP	OSP*SP	OP1	0	↑	0
25	↓	0	0	OP1*OSP	OSP*SP	OP1	↓	0	↓
26	↑	0	0	OP1*OSP	OSP*SP	OP1	↑	0	↑
27	0	0	0	OP1*OSP	OSP*SP	OP1	0	0	0

OP1: Osmostress induced pathway 1; OSP: Osmostress induced STATc pathway; SP: STATc pathway irrespective of osmostress; wt: AX2 wild type cells; ko: knock-out; RIC: random integrant cells; ↑ and/or red: up-regulated; ↓ and/or blue: down-regulated; 0 and/or black: non-regulated; +: treated; -: untreated

3.3.3 Two clusters define STATc-regulated genes

Of the 741 differentially regulated genes that were common between two or three of the above comparisons three genes were removed because of missing values in one of the experiments and the remaining 738 genes were subjected to cluster analysis. This way we could reduce the 27 possible cases from table 8 to eight major outputs or clusters (Figure 31). Cluster 1 houses those genes that were down-regulated by OP1 (Fig 31, III) but up-regulated by OSP and/or SP (Figure 31, II). The regulatory outcome of treated versus untreated wt cells (Figure 31, I) depended on the balance between OP1 and the regulation by STATc. Cluster 2 is comprised of genes that were down-regulated by OP1 and their regulation was found to be largely independent of STATc (Figure 31, I to III). Cluster 3 genes were up-regulated by OP1 (Figure 31, III) and down-regulated by STATc through OSP and/or SP (Figure 31, II); the OP1 response dominated the regulatory output in the experiment with treated versus untreated wt cells (Figure 31, I). Interestingly, the up-regulation for genes in this cluster was weaker with wt cells than with STATc knock-out cells (compare Figure 31, I and III). This suggests that OP1 and STATc act oppositely on these genes. Cluster 4 is characterized by genes that are down-regulated by STATc and either up-regulated or non-affected by OP1 (Figure 31, II and III). STATc dominates the regulatory output because the differential expression in the experiment with treated versus untreated wt cells is similar to the experiment with treated RIC versus treated STATc knock-out cells (Figure 31, I and II). Cluster 5 is a very small cluster with genes that are slightly down-regulated by OP1 and OSP (Figure 31, I to III). The last three clusters comprise genes that were up-regulated in treated versus untreated wt cells. Cluster 6 genes were up-regulated by OP1 and either unaffected or slightly up-regulated by STATc, while cluster 7 genes were up-regulated by STATc and either unaffected or slightly up-regulated by OP1 (Figure 31, I to III). Finally, cluster 8 genes were up-regulated by OP1 and OSP (Figure 31, I to III). Particularly interesting were the cluster 4 and 7 genes, which were regulated by STATc and this regulation also dominated the transciptional output of wt cells (experiment I). They constitute approximately 20% of the genes that were common between two or three experiments.

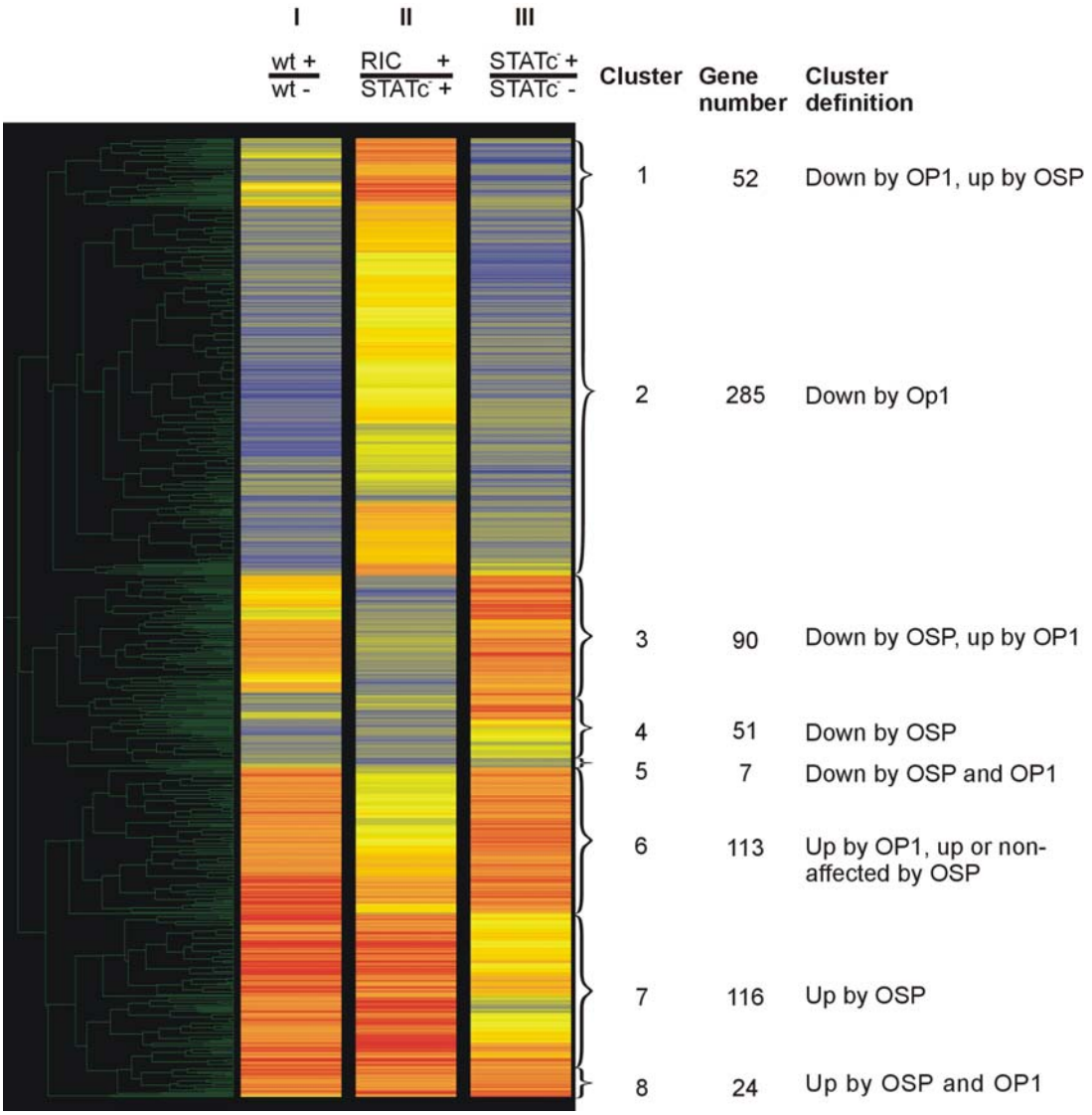

Figure 31. Cluster analysis reveals clusters of STATc-regulated genes. Shared genes of figure 28 were clustered with GeneSpring 7.2. Eight major clusters (1-8) can be distinguished of which clusters 4 and 7 contain those genes that are solely regulated by STATc. The dendrogram is displayed on the left. The differentially regulated genes are depicted as coloured lines. The colour represents the fold of induction (red) or repression (blue) (colour scale see figure 15). Non-regulated genes are displayed in yellow. OP1: Osmostress induced pathway 1; OSP: Osmostress induced STATc pathway; SP: STATc pathway irrespective of osmostress.

To learn more about these STATc-regulated genes we subjected them to GO analysis. For the down-regulated genes in cluster 4 we found an enrichment of the biological process terms biosynthesis, proton transport and coenzyme metabolism, in particular ATP metabolism. The up-regulated genes in cluster 7 are characterized by an enrichment of the biological process terms response to osmotic stress, nitrogen compound metabolism, endosome organization and biogenesis and actin filament based process. The full lists of enriched biological process, molecular function and cellular component terms are available as supplementary information (Table SI 2). The results of the comparisons clearly show that STATc is the responsible regulator

for a subset of the differentially regulated genes in the *Dictyostelium* osmostress response. Apparently most of the up-regulated genes in the osmotic response, which encode cytoskeletal proteins are subject to regulation by STATc. This holds also true for a subset of those genes that are involved in metabolism or the response to stress or are responsible for ion homeostasis (Figure 32). These results also imply that at least two signaling pathways get activated in *Dictyostelium* cells in response to hyperosmotic stress.

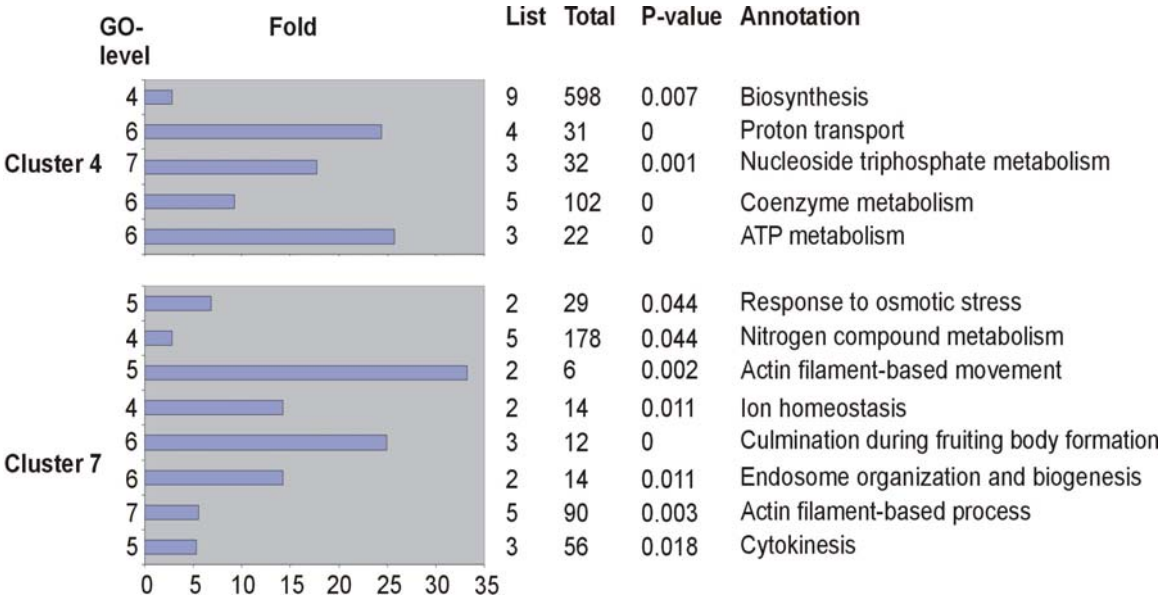

Figure 32. GO biological process terms enriched in cluster 4 and 7. See figure 16 for figure legend.

Manual annotation revealed that the down-regulation of many vacuolar H^+-ATPase subunits is dependent on STATc, including *vatA*, *vatC*, *vatE*, *vatM* and putative *vatF* (Table 10). The up-regulation of many cytoskeletal genes is also dependent on STATc, including *abnB* (actobindin), *sevA* (severin), *ctxA* (cortexillin I), *ctxB* (cortexillin II), *dct* (dynacortin), *limD* (LIM domain-containing protein), *forA* (formin homology domain-containing protein), *myoA* (myosin IA heavy chain) and *myoK* (myosin IK heavy chain). It is apparent that many genes involved in metabolism are induced by STATc, which suggests that STATc significantly regulates part of the metabolic machinery of *Dictyostelium* in response to hyperosmotic shock. The up-regulation of some of these genes (e.g. *allC*, *tpsA*, *tpsB*, *celB*, and genes encoding Cellobiohydrolase I, pyrroline-5-carboxylate reductase, Alpha amylase, uricase) could be required for the synthesis of osmolytes. The dependence of three SrfA-induced genes (*sigG*, *sigI*, *sigJ*) on STATc further rules out a role of SrfA in the

osmostress response. Interestingly the induction of *ptpC* encoding the STATc tyrosine phosphatase (J. Williams, personal communication) is also dependent on STATc (Table 11).

Table 10. Annotation of STATc down-regulated genes in cluster 4

DDB ID	Annotation	Differential Expression		
		I	II	III
DDB0167206	***hspK***, heat shock protein Hsp20 domain-containing protein	0.250	0.561	1.010
DDB0169046	***HspG7***, heat shock protein Hsp20 domain-containing protein	0.486	0.366	5.295
DDB0169207	***HspG12***, heat shock protein Hsp20 domain-containing protein	0.587	0.647	2.617
DDB0185092	***cprE***, cysteine proteinase 5 precursor	0.607	0.569	0.900
DDB0169112	NAD-dependent epimerase/dehydratase family protein	0.718	0.370	2.887
DDB0168979	Member of the Major Facilitator Superfamily (MFS)	0.283	0.367	4.768
DDB0169506	***gtr2***, alpha amylase domain-containing protein, starch synthase-like protein, might be involved in glycogen biosynthesis, GlycosylTRansferase	0.620	0.592	0.819
DDB0183840	***grlE***, G-protein-coupled receptor (GPCR) family protein	0.649	0.765	1.659
DDB0201563	***vatA***, vacuolar H+-ATPase A subunit	0.473	0.630	0.779
DDB0191419	***vatC***, H(+)-transporting ATPase	0.368	0.599	0.871
DDB0185070	***vatE***, vacuolar H+-ATPase E subunit	0.442	0.656	1.072
DDB0216215	***vatM***, vacuolar proton ATPase 100-kDa subunit	0.566	0.552	1.052
DDB0216933	Similar to H+-transporting ATPase	0.445	0.616	0.805
DDB0191505	***vacA***, vacuolin A	0.482	0.644	1.294
DDB0188183	pantoate-beta-alanine ligase, pantothenate biosynthesis, alanine related	0.755	0.408	2.048
DDB0188843	Physaropepsin.	0.540	0.556	0.717
DDB0191201	***cahA***, carbonic anhydrase	0.627	0.537	0.952
DDB0191230	***AbcG3***, ABC transporter G family protein	0.425	0.807	1.869
DDB0191399	***GchA***, GTP cyclohydrolase I	0.427	0.433	0.598
DDB0192069	***slc25a3***, phosphate carrier protein, mitochondrial substrate carrier family protein	0.462	0.508	0.968
DDB0206429	Peptidase C1A family protein	0.545	0.604	1.108
DDB0215000	***forB***, actin binding protein	0.565	0.605	0.692
DDB0215380	***ponA***, ponticulin	0.588	0.658	1.619
DDB0215363	***alrA***, aldehyde reductase	0.640	0.666	3.127
DDB0215391	***rps2***, ribosomal protein S2	0.731	0.599	1.164
DDB0230025	***rps8***, 40S ribosomal protein S8	0.586	0.613	0.900
DDB0230068	beta-ketoacyl synthase family protein, Lipid biosynthesis	0.467	0.492	0.895
DDB0231286	***alrE***, aldo-keto reductase	0.461	0.637	3.608
DDB0231294	***idhB***, isocitrate dehydrogenase (NAD+), isocitrate dehydrogenase (NAD+) beta subunit	0.587	0.436	0.896
DDB0231311	***maspS***, aspartyl-tRNA synthetase	0.611	0.636	1.798
DDB0231429	putative glutathione transferase	0.553	0.691	2.981

Table 11. Annotation of STATc up-regulated genes in cluster 7

DDB ID	Annotation	Differential Expression		
		I	II	III
DDB0183957	***gnrB***, gelsolin-related protein	1.756	2.108	0.919
DDB0185034	***csbA***, contact sites B protein, Cell adhesion	1.903	5.306	0.973
DDB0185093	***csbB***, contact sites B protein, Cell adhesion	1.880	4.249	1.109
DDB0190330	***AbnB***, actobindin	1.923	9.515	1.645
DDB0188380	***sevA***, severin	1.968	1.635	1.287
DDB0191103	***ctxA***, actin binding protein	2.396	5.184	0.952
DDB0185031	***ctxB***, cortexillin II	1.932	3.971	0.589
DDB0191252	***dct***, dynacortin	3.523	4.033	0.915
DDB0201567	***limD***, LIM domain-containing protein	1.580	2.135	1.063
DDB0214996	***forA***, actin binding protein	2.105	2.037	1.082
DDB0215392	***myoA***, myosin IA heavy chain	1.746	2.102	0.946
DDB0185086	***myoK***, myosin IK, unconventional myosin heavy chain	1.772	2.070	1.012
DDB0185106	***ionA***, Sodium/potassium-transporting ATPase alpha-4 chain	1.592	2.123	0.982
DDB0185120	***rtoA***, unknown	15.271	10.184	0.996
DDB0185328	***sodB***, superoxide dismutase	2.736	3.203	0.707
DDB0185565	***cbpB***, calcium-binding protein	1.891	1.879	1.058
DDB0185907	***slc25a11***, mitochondrial 2-oxoglutarate/malate carrier protein	2.723	2.293	1.094

DDB ID	Annotation	Differential Expression		
		I	II	III
DDB0219248	***tpsA***, glycosyltransferase, treholose biosynthesis	4.913	2.541	0.989
DDB0186292	***tpsB***, glycosyltransferase, treholose biosynthesis	2.706	1.706	1.011
DDB0187157	IPT/TIG domain-containing protein	2.116	1.501	0.902
DDB0187698	DENN domain-containing protein	1.730	2.019	0.869
DDB0188438	P-type ATPase, Ca2+-ATPase	2.035	1.569	1.188
DDB0188646	Serine:pyruvate/alanine:glyoxylate aminotransferase	1.628	2.567	0.695
DDB0190241	Pyrroline-5-carboxylate reductase, PCA reductase	7.508	4.943	2.004
DDB0191109	***pmpA***, putative membrane protein	2.663	3.698	1.747
DDB0191112	***tipC***, vacuolar protein sorting-associated protein	3.753	1.794	1.037
DDB0202233	Cellobiohydrolase I	9.796	3.112	1.064
DDB0191122	***celB***, cellulose binding protein	15.335	2.736	1.297
DDB0191171	***cplA***, calpain-like cysteine protease	1.968	3.102	0.931
DDB0191266	***mftA***, mitochondrial substrate carrier family protein	2.166	1.720	1.218
DDB0191348	***pgmA***, phosphoglucomutase A	1.535	1.804	0.944
DDB0191392	***SigG***	6.503	11.017	1.210
DDB0191353	***sigI***, CBS (cystathionine-beta-synthase) domain-containing protein	6.958	3.480	1.232
DDB0191111	***SigJ***	27.650	5.931	2.049
DDB0191437	***rgaA***, RasGTPase-activating protein	1.789	4.964	0.796
DDB0191769	***rtnlc***, reticulon family protein	2.446	2.219	1.192
DDB0203727	antioxidant enzyme	10.862	8.855	1.024
DDB0204016	***gpt10***, putative glycophosphotransferase	1.667	1.881	1.077
DDB0204785	***prlA***, proliferation associated protein, highly conserved metalloexopeptidase, proteolysis	2.047	2.928	0.962
DDB0204945	Alpha amylase family protein, oligosaccharide degradation	4.324	3.823	0.982
DDB0205008	Acetylornitine deacetylase, lysine biosynthesis	2.861	1.971	1.287
DDB0206314	putative transmembrane protein	3.618	2.799	1.276
DDB0214826	***racI***, Rho GTPase	2.427	2.799	0.955
DDB0214986	***ptpC***, protein tyrosine phosphatase	1.928	2.157	1.055
DDB0191116	***dstB***, STATfamily protein	1.751	1.936	1.047
DDB0215378	***dstC***, STATfamily protein	4.741	8.841	0.509
DDB0217489	WD40-like domain-containing protein	1.951	1.588	0.762
DDB0217773	mitochondrial substrate carrier family protein	3.754	2.618	1.033
DDB0217917	doublecortin domain-containing protein	1.673	2.133	1.029
DDB0218131	CBS (cystathionine-beta-synthase) domain-containing protein	3.723	1.629	1.011
DDB0218638	***aass***, aminoadipic semialdehyde synthase, NAD+, L-glutamate-forming	2.376	2.428	1.117
DDB0230093	***aatB***, aspartate aminotransferase, aspartate degradation	2.406	2.834	1.366
DDB0231199	MORN repeat-containing protein kinase	2.007	1.739	1.021
DDB0231436	glutathione S-transferase domain-containing protein	3.107	3.169	1.119
DDB0231470	uricase, urate oxidase	2.305	5.841	1.226
DDB0231471	***allC***, allantoicase, urate degradation	8.597	7.372	1.537

3.4 Generation of knock-out mutants of putative STATc protein kinase

3.4.1 Possible Janus kinases of STATc in *Dictyostelium*

In vertebrate, STAT is tyrosine phosphorylated and activated by an upstream Janus kinase (JAK) upon extracellular stimuli (Darnell et al., 1994). So far JAK has not been reported in *Dictyostelium*. However, the activity is present since STATc is tyrosine-phosphorylated during sorbitol treatment for 15 minutes followed by nuclear translocation (Araki et al., 2003). We used two strategies to identify the possible tyrosine kinase of STATc, BLAST search and microarray analysis. The latter

approach assumed that STATc tyrosine kinase is up-regulated in our experimental time scale, and the induction might be dependent on STATc.

For the BLAST search, we used mouse JAK1 (NCBI accession P52332) to blast against all *Dictyostelium* proteins. The best hits are listed in Table 12. In support of the BLAST result, the expression values show that the induction of DDB0231199 is completely dependent on STATc and that of pkyA is partially dependent on STATc (Table 11). Therefore *pkyA* and DDB0231199 were chosen for further investigation.

Table 12. Possible JAK1 homologues in the *Dictyostelium* genome

DDB ID	Annotation	E-value	Differential Expression		
			WT treated vs WT untreated	RIC treated vs STATc- treated	STATc- treated vs STATc- untreated
DDB0191218	***pkyA***, tyrosine kinase-like protein	1E-34	1.60	1.11	1.15
DDB0214883	***drkD***, tyrosine kinase-like protein	4E-27	1.18	0.63	1.10
DDB0229963	tyrosine kinase-like protein	3E-24	N/A	N/A	N/A
DDB0231199	tyrosine kinase-like protein	2E-23	2.01	1.74	1.02
DDB0230060	***drkA***, tyrosine kinase-like protein	1E-22	0.76	1.02	0.95

Differentially regulated genes are highlighted in red.

3.4.2 Domain architecture of JAK1, PkyA and DDB0231199

Mouse JAK1 is a protein of 1153 amino acids, containing a plasma membrane-binding domain B41, an Src homology 2 (SH2) domain, a possible dual-specificity Ser/Thr/Tyr kinase domain (STYKc) and a tyrosine kinase domain (TyrKc). The first kinase domain is probably non-functional and the second tyrosine kinase domain is the active catalytic domain. *PkyA* and DDB0231199 encode tyrosine kinase-like proteins of 1338 and 1634 amino acids, respectively. The latter contains three MORN (Membrane Occupation and Recognition Nexus) repeats at the N-terminal, which are responsible for the recruitment of the protein to the plasma membrane (Figure 33).

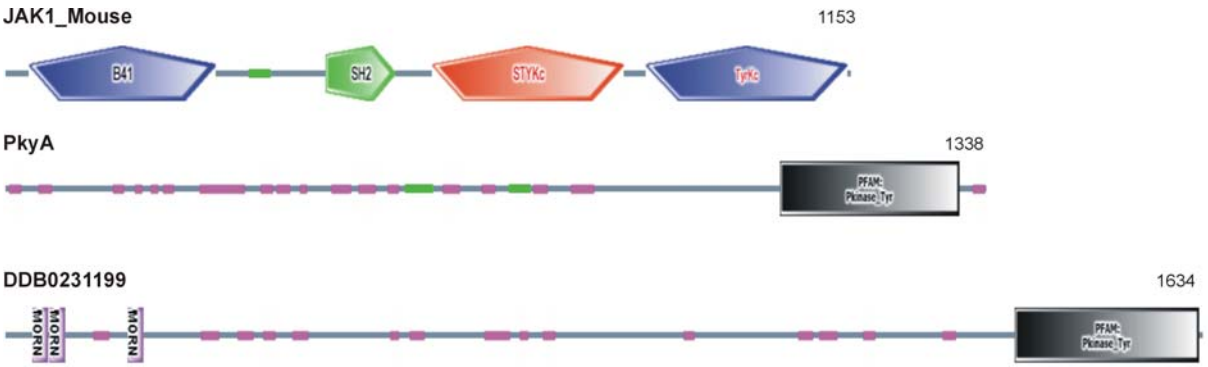

Figure 33. Schematic representation of mouse JAK1, and PkyA and DDB0231199 from *Dictyostelium*. Domain structure was predicted using SMART (http://smart.embl-heidelberg.de/). B41 stands for band 4.1 homologues, a plasma membrane-binding domain, SH2 for Src homology 2 domain, STYKc for possible dual-specificity Ser/Thr/Tyr kinase domain, TyrKc and Pkinase_Tyr for tyrosine kinase domain and MORN for membrane occupation and recognition nexus domain.

3.4.3 *PkyA* and DDB0231199 gene replacement by homologous recombination

A targeting vector was constructed [see 2.2.3.7] that allowed replacement of a 2.37 kb fragment of the *pkyA* gene (Figure 34). The strategy was such that most part of exon 1 was replaced with the blasticidin resistance cassette (1.35 kb), thus disrupting a part of the Pkinase_Tyr domain. The targeting fragment was transfected into wild type AX2 cells by electroporation.

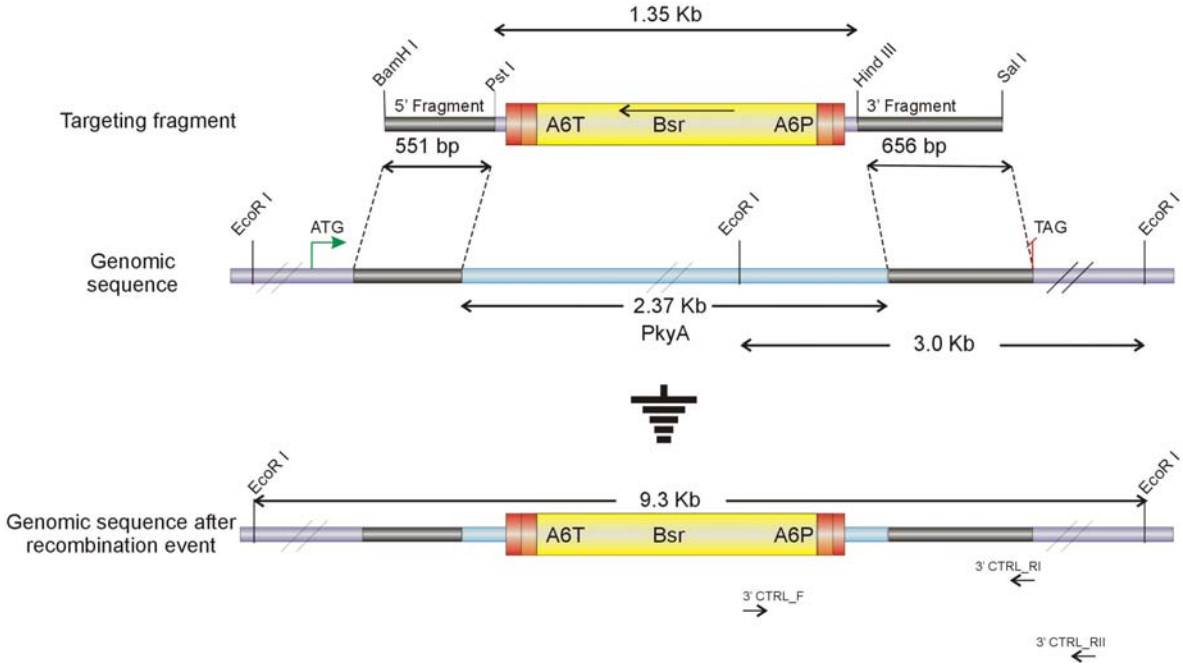

Figure 34. Strategy for the replacement of the *pkyA* gene. Part of the targeting vector is shown on top, the genomic sequence before recombination in the middle and after the recombination event at the bottom. Shown in red colour are the stop codons, six upstream of the Actin-6 promoter and one upstream the Actin-6 terminator. Displayed in orange are the Cre-*loxP* recombination sites. Primers for PCR screening are displayed as arrows. The 3' fragment (656 bp) shown in black was used as probe for the Southern blot. A6P: Actin 6 promoter. A6T: Actin 6 terminator. Bsr: blasticidin resistance cassette. The illustration does not reflect the true scale of actual parts of the disruption vector.

Similarly, a targeting vector was constructed that allowed replacement of a 3.73 kb fragment of the gene DDB0231199 (Figure 35). The targeting fragment consisted of the blasticidin resistance cassette and part of exon 2 and exon 3, thus disrupting the Pkinase_Tyr domain. The targeting fragment was transfected into wild type AX2 cells by electroporation.

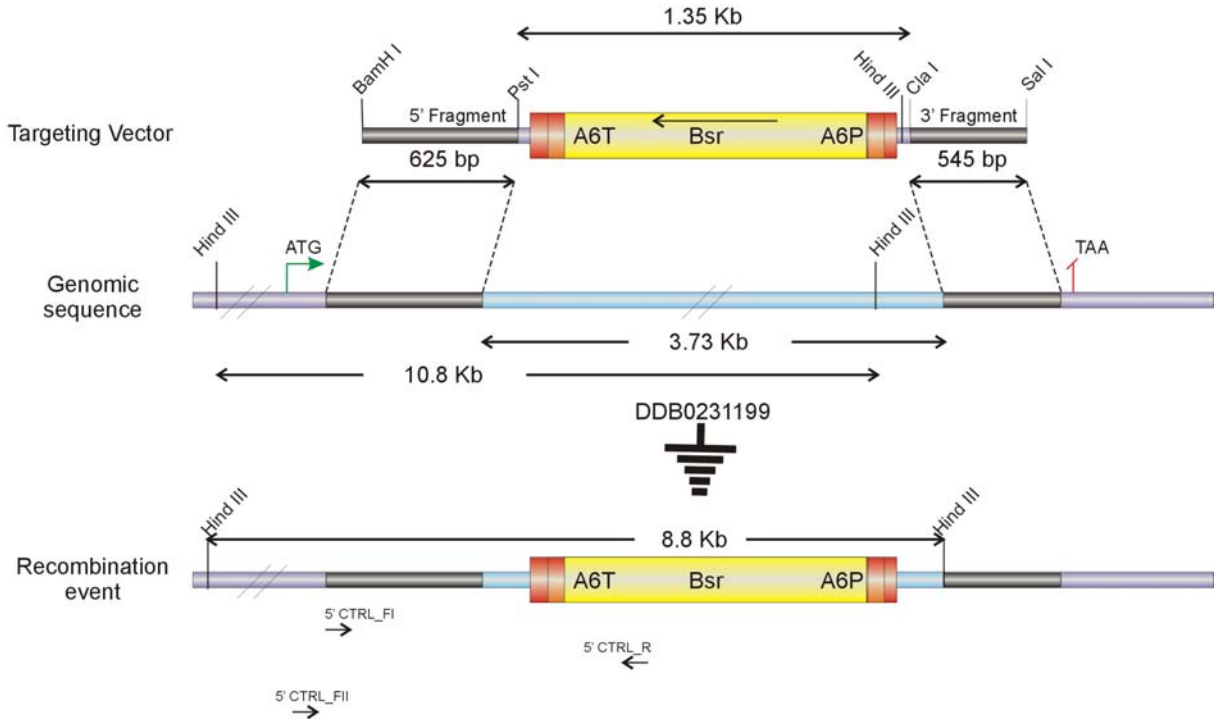

Figure 35. Strategy for the replacement of the DDB0231199 gene. The illustration depicts the strategy for DDB0231199 gene replacement. Part of the targeting vector is shown on top, the genomic sequence before recombination in the middle and after the recombination event at the bottom. Shown in red colour are the stop codons, six upstream of the Actin-6 promoter and one upstream the Actin-6 terminator. Displayed in orange are the Cre-*loxP* recombination sites. Primers for PCR screening are displayed as arrows. The 5' fragment (625 bp) shown in black was used as probe for the Southern blot. The illustration does not reflect the true scale of actual parts of the disruption vector.

3.4.4 Confirmation of homologous recombination by PCR and Southern blot analysis

To investigate blasticidin-resistant clones, PCR and Southern blot analysis were carried out.

Genomic DNA isolated from wild type AX2 cells and transformants was used as template for PCR. For screening possible *pkyA* mutants, the forward primer (3' CTRL_F) located at the 5' end region of the bsr gene and two reverse primers (3' CTRL_RI and 3' CTRL_RII) located at the 3' end of the gene were used. In case of a correct recombination event, this should lead to PCR products with a size of 1.1 kb and 1.4 kb, respectively. No PCR product was obtained from AX2 wild type genomic DNA (Figure 36A).

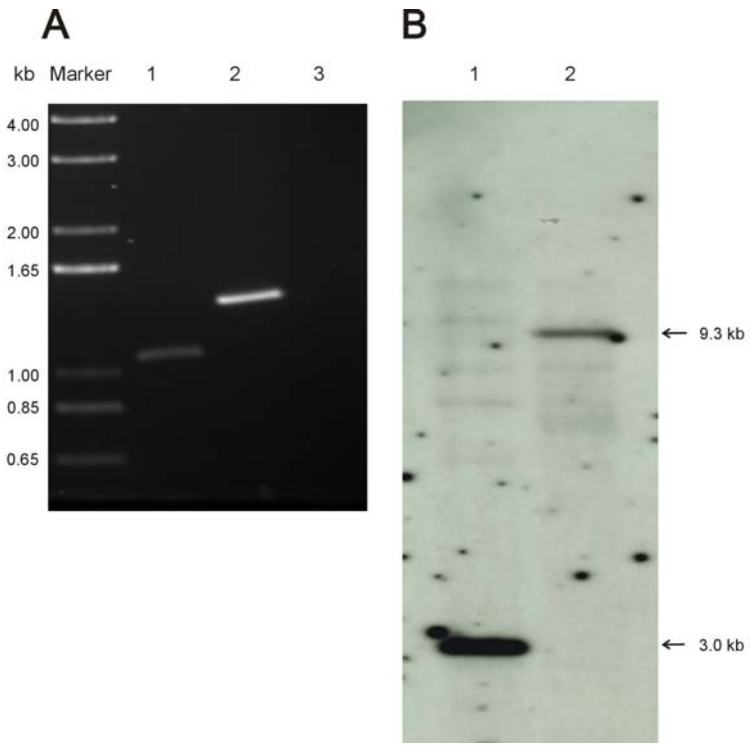

Figure 36. PCR screening and Southern blot analysis of the *pkyA* knock-out mutant. A) PCR screening with primers 3' CTRL_F and 3' CTRL_RI (lane 1) and 3' CTRL_F + 3' CTRL_RII (lane 2), yielded the expected products of 1.1 and 1.4 kb, respectively, in the case of the mutant. No product was obtained in the case of wild type AX2 cell (lane 3). B) Southern blot analysis: As expected a band of 3.0 kb in the case of AX2 wild type (lane 1) and of 9.3 kb in the case of the mutant (lane 2) were obtained.

Positive transformants were further confirmed by Southern blot. Genomic DNA was digested with *Eco*RI and probed with the 3' probe [see Figure 34]. The *Eco*RI restriction site was replaced by the targeting fragment in the case of homologous recombination thus leading to a fragment of 9.3 kb. In the wild type situation a fragment of 3.0 kb was obtained as expected (Figure 36B).

Similarly in the case of gene DDB0231199, two forward primers (5' CTRL_FI and 5' CTRL_FII) located at the 5' end of the gene DDB0231199 and the reverse primer (5' CTRL_R) located at the 3' end of bsr gene were used for screening. In the case of homologous recombination event, this should lead to PCR products of 1.4 and 1.5 kb, respectively. No PCR product was obtained from AX2 wild type genomic DNA (Figure 37).

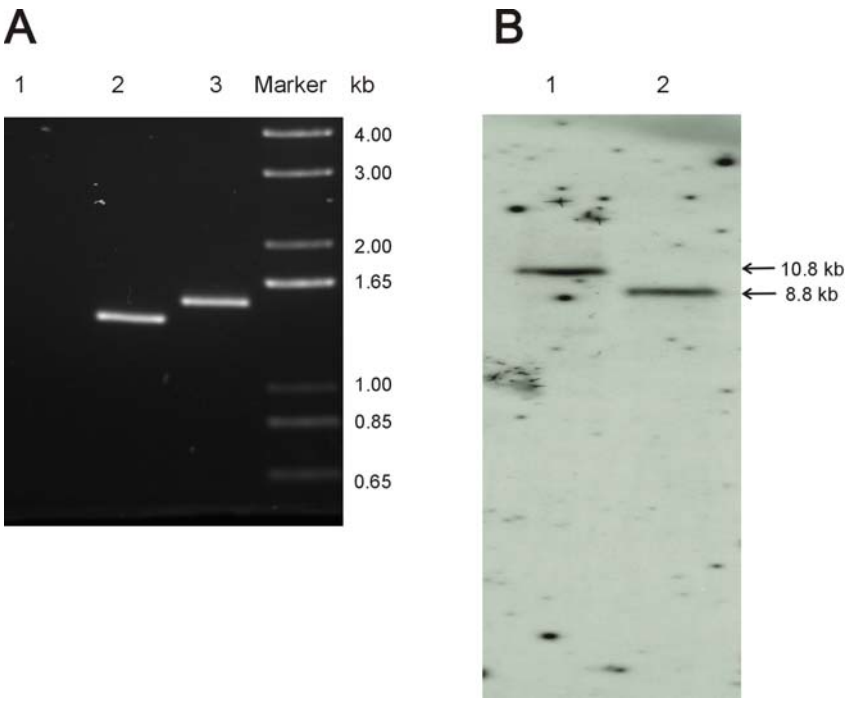

Figure 37. PCR screening and southern blot analysis of the DDB0231199 knock-out mutant. A) PCR screening with primers 5' CTRL_FI and 5' CTRL_R (lane 2) and 5' CTRL_FII and 5' CTRL_R (lane 3) yielded the expected the expected products of 1.4 and 1.5 kb, respectively, in the case of the mutant. No product was obtained in the case of wild type AX2 cell (lane 1). B) Southern blot analysis: a band of 10.8 kb in the case of AX2 wild type (lane 1) and of 8.8 kb in the case of the mutant (lane 2) were obtained.

Positive transformants were further confirmed by southern blot. Genomic DNA was digested with *Hind*III and probed with the 5' probe [see Figure 35]. The endogenous *Hind*III restriction site was replaced by the targeting fragment and a new *Hind*III restriction site was introduced in the case of homologous recombination. This resulted in a fragment of 8.8 kb in the mutant and of 10.8 kb in wild type AX2 cells (Figure 37).

Further characterization of *pkyA* and DDB0231199 knock-out mutants is currently underway.

4 Discussion

Dictyostelium is a powerful model system for large-scale studies of the transcriptional and translational adaptations to a changing osmotic environment. The organism is amenable to genetic manipulation, the complete genome has recently been sequenced and cDNA microarrays for global transcriptional analyses are available (Eichinger, 2003; Eichinger et al., 2005; Kaul and Eichinger, 2006; Shaulsky and Loomis, 2002). We have used these advantages of *Dictyostelium* to study its response to hyperosmotic conditions after one hour of exposure to sorbitol, in a time course experiment and by comparing the transcriptional profiles of treated or untreated wild type with SrfA and with STATc knock-out cells, respectively. We have also generated knock-out mutants of two candidate STATc protein kinases that await further characterization.

4.1 Reliability of the microarray results

As a powerful tool to investigate gene expression, cDNA microarrays have had a profound impact on biological research over the last decade. Microarray results need to be reliable as they are used for the generation of hypotheses that constitute the basis for future work. Therefore, the quality control of the used microarrays and the applied analyses are crucially important.

The *Dictyostelium* cDNA microarray is produced in house, and the quality of the microarray results is guaranteed by different means. Firstly, the SpotReport® −10 Array Validation System as well as appropriate positive and negative controls are present on the DNA microarray (GEO; http://www.ncbi.nlm.nih.gov/geo; accession number GPL1972), thus allowing to test for sensitivity and linearity of the results. Secondly, LOWESS-normalization is used to correct a possible colour bias that might be generated during the experiment (Figure 13). Thirdly, differentially regulated genes are detected by Significance Analysis of Microarrays (SAM) (Figure 14). SAM calculates a score for every gene with a t-statistic, modified for the use on microarray data. The higher the score the more reliable is the differential expression of the reported gene. This statistic is superior to a fold change cut-off or a t-test, as was shown with Northern blots (Jones and Arvin, 2003) and a simulation (Smyth et al., 2003). As very low folds of change cannot be confirmed with alternative methods and

the biological impact of such small changes is unclear, an additional threshold for the fold change of 1.5 was used for the analysis of all experiments.

Consistent with recent research (Shi, 2006), our microarray data showed much lower standard deviation than real time PCR data (Figure 15). However, the fold change of the microarray data was compressed as compared to real time PCR and Northern blot data for the genes tested (Figure 15 and 16). The phenomenon is well known, its exact cause is, however, not clear.

We performed a stringent test of our experimental settings by comparing untreated cells with untreated cells at t_0 of the time course. The results showed no differentially regulated gene at the threshold of 1.5 (Table 1 and Figure 17), and thus confirmed the reliability of our experimental settings.

4.2 A variety of cellular responses in *Dictyostelium* cells indicates a complex defence mechanism to hyperosmotic conditions

Treatment of *Dictyostelium* cells with 200 mM sorbitol resulted in dramatic transcriptional changes. In the time course experiment more than 800 unique genes were differentially regulated. A cluster analysis revealed four major clusters. Clusters 1 and 3 were characterized by up-regulated and clusters 2 and 4 by down-regulated genes (Figure 17). Several cellular systems seem to be important for the stress response.

4.2.1 Cytoskeleton

Upon sorbitol treatment, *Dictyostelium* cells shrank immediately (Figure 10), actin was tyrosine-phosphorylated (Figure 8) and the actin cytoskeleton was reorganized thus leading to the formation of a stringent cortex (Figure 9). In agreement with the cellular changes many cytoskeletal genes were differentially regulated and found to be enriched in clusters 2, 3 and 4 (Table 3-5).

Actin tyrosine phosphorylation has been reported to inhibit filament nucleation and elongation and to destabilize actin filaments (Liu et al., 2006). The possible mechanism is that tyrosine phosphorylation locks the DNase I-binding loop in a conformation that inhibits ATP hydrolysis accompanying polymerization and also weakens actin-actin interactions, particularly at the pointed end. The latter could inhibit nucleation, increase the critical concentration at the pointed end, inhibit interaction between actin and Arp2/3-VCA (verpolin, cofilin, and acidic domains of

human wASP) or other proteins, and destabilize filaments. The differential regulation of genes involved in actin nucleation, including *arpB* (actin related protein 2), *forA* (formin A), *forB* (formin B), *forF* (formin F), *ponA* (ponticulin), *proA* (profilin I) and *proB* (profilin II), indicates a long term reorganization of the actin cytoskeleton. We also found that *mhcA* (myosin II heavy chain) is upregulated. During osmotic shock, myosin II becomes phosphorylated on both heavy and light chains. The heavy chain phosphorylation is associated with destabilization of existing filaments, and could therefore allow myosin II to mobilize and relocalize (Insall, 1996).

Thus, on the level of the actin cytoskeleton two effects are observed, short term effects i.e. phosphorylation of actin and myosin II and long term effects i.e. differential regulation of a number of cytoskeletal genes.

4.2.2 Metabolism

The enrichment of GO terms in clusters 2 and 4 showed a down-regulation of metabolic processes (Figure 20 and 24). The overall down-regulation of the metabolic machinery seems to be a negative strategy of *Dictyostelium* to overcome this unfavourable condition. In particular the metabolism of carbohydrates, amino acids and nucleotides appear to be affected (Figure 26-29). In addition, we also found a number of up-regulated genes involved in metabolism in cluster 3 (Table 4), which were not reported by GOAT. Some of these could be involved in the production of osmolytes. Further annotation of the genes involved in metabolic pathways suggests a reprogramming of the metabolic machinery, leading to organic osmolytes accumulation, including trehalose, urea and amino acids (Figure 38).

4.2.2.1 Do *Dictyostelium* cells synthesize a compatible osmolyte?

Schuster et al. analyzed *Dictyostelium* cells exposed to 400 mM sorbitol and found that no accumulation of naturally occurring amino acids and glycerol (Schuster et al., 1996). Zischka et al. measured the osmolarity of cell lysates after 400 mM sorbitol treatment and found that the increase of intracellular osmolarity is not sufficient to counteract the external forces. Therefore, they claimed that *Dictyostelium* does not produce compatible osmolytes upon hyperosmotic shock (Zischka et al., 1999). However, we found that *Dictyostelium* cells were able to adapt to 100 mM sorbitol after about 1 hour, indicating the production of osmolytes to counterbalance

the extracellular osmolarity. Based on our expression data, there are three obvious candidates for organic osmolyte: trehalose, amino acids and urea.

Trehalose is a disaccharide of glucose that is predominantly used in bacteria, fungi (including yeasts), plants, and invertebrates. It is a general stress protectant and assists chaperones in controlling protein denaturation and renaturation (Francois and Parrou, 2001; Singer and Lindquist, 1998a; Singer and Lindquist, 1998b). Vegetative *Dictyostelium* cells store 0.2 mmol glycogen per kg dry weight and other polysaccharides (Rutherford, 1976; Yamada et al., 1974), whose degradation could provide a significant amount of glucose. A role of trehalose as compatible osmolyte in *Dictyostelium* cells is supported by the up-regulation of the enzyme tpsA that is responsible for the conversion of glucose to trehalose (Figure 26). In addition, we found the down-regulation of four genes related to counting factor CF (*cf45-1*, *cnrI*, *ctnA*, and *smlA*). CF regulates group size during development and is important for glucose homeostasis: cells lacking bioactive CF have high glucose levels, whereas transformants oversecreting CF have low glucose levels (Jang et al., 2002). The down-regulation of counting factor related genes might therefore contribute to a rise in glucose, which can be converted to trehalose. Their differential regulation also constitutes an example for genes involved in development and osmostress response (see 4.2.5).

Steck et al. showed that *Dictyostelium* cells have a large cytoplasmic pool (50 mM) of a few small, neutral amino acids, predominantly glycine, alanine and proline. Their excretion was decreased in response to hyperosmotic shock, and the abundance of several additional amino acids, including tyrosine, methionine, valine, leucine, isoleucine and phenylalanine, rose several-fold (Steck et al., 1997). In addition, Zischka et al. showed that hyperosmotic stress induced ubiquitnation of cellular proteins (Zischka et al., 1999). We found an up-regulation of genes involved in protein degradation, and differential regulation of genes involved in amino acids metabolism (Figure 28). This cellular response could explain the accumulation of amino acids.

Another candidate for the organic osmolyte is urea. Urea is a small organic compound, often considered a metabolic waste product, which at high concentrations is known to have deleterious effects on protein structure and function. However, there are several examples of vertebrate tissues and cells that accumulate urea to high concentrations (Yancey, 1994). One of them, marine elasmobranch fishes,

accumulate urea to concentrations as high as 300–500 mmol/l (Ballantyne, 1997; Yancey et al., 1982; Yancey, 1994). We found that three genes encoding the key enzymes for purine degradation were highly up-regulated, including AMP deaminase, uricase and allantoicase. They might contribute to a rise in urea. So far there is no direct biochemical evidence for the accumulation of urea in *Dictyostelium* upon hyperosmotic stress. However, it was reported that urea is accumulated to 2% of the dry weight of cells during development (Payne, 2005) (see also 4.2.5). It is also notworthy that *Dictyostelium* does not have a functioning urea cycle, which plays an important role on the synthesis of urea in human (Payne, 2005).

In support of these metabolic changes we also found differential regulation of several transporter genes. For example, the member of Major Facilitator Superfamily (MFS) gene was significantly down-regulated so that the efflux of small solutes might be decreased. In contrast, the sodium/potassium-transporting ATPase was up-regulated which could lead to an increase in intracellular K^+ (Figure 38).

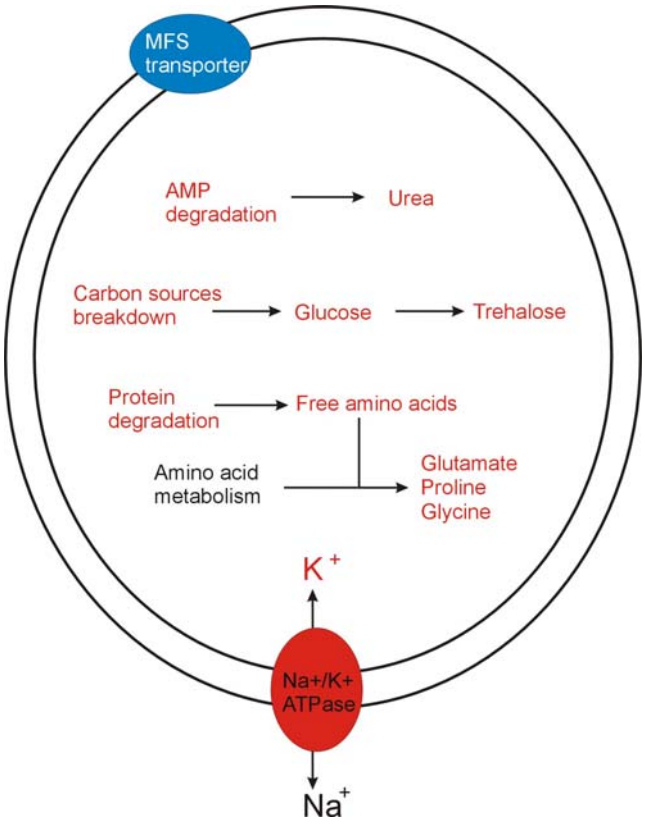

Figure 38 Possible strategies of *Dictyostelium* to accumulate organic and inorganic solutes. Urea might be accumulated through AMP degradation, trehalose might be synthesized from glucose and protein degradation could lead to an increase of free amino acids. Differential regulation of genes involved in amino acid metabolism could further increase the concentration of amino acids, in particular glutamate, proline, glycine, alanine. An up-regulation of the Na^+/K^+ ATPase could lead to an increase in intracellular K^+ concentration, that could act as counterion for glutamate. The Major Facilitator Superfamily (MFS) gene was significantly down-regulated so that the efflux of small solutes might be decreased.

Future biochemical experiments should reveal the contributions of these candidate osmolytes *Dictyostelium* osmoprotection.

In summary, the strategies of *Dictyostelium* to accumulate organic and inorganic solutes might be very similar to the ones from *E. coli* (Figure 5 and 38). We assume that *Dictyostelium* uses a mixture of different osmolytes and protective mechanisms in response to hypertonicity. The supposed metabolic changes appear more similar to *E. coli* than to *S. cerevisiae* (Hohmann, 2002; Wood, 2006).

4.2.3 Antioxidant system

Osmotic stress demands metabolic adjustments and in general requires the cell to invest energy to cope with the consequences of cell damage and to produce protective proteins or metabolites. This in turn leads to the production of reactive oxygen species (ROS) and the change of the redox state in the cell. Hence, the redox metabolism needs to be adjusted (Hohmann, 2002). Koziol et al. demonstrated that hypertonicity increased the generation of superoxide and other reactive species in yeast cells. In addition, yeast mutants lacking superoxide dismutase were more sensitive than wild-type cells to osmotic stress (Koziol et al., 2005).

We found that the expression of a remarkable number of genes encoding enzymes involved in the defense from oxidative damage and in redox metabolism was stimulated in hyperosmotic conditions. Examples of such genes include grxA (glutaredoxin), DDB0203727 (antioxidant enzyme), sodB (superoxide dismutase) and DDB0231436 (glutathione S-transferase domain-containing protein). However, the level of up-regulation of these genes was different: the up-regulation of the antioxidant enzyme and sodB was much higher than that of grxA (glutaredoxin) and of DDB0231436 (glutathione S-transferase domain-containing protein). The antioxidant enzyme might control the peroxide levels while SodB should be responsible for the removal of superoxide (O_2^-) radicals (Bloomfield and Pears, 2003; Claiborne et al., 1999). However, we also found three genes (DDB0231429, DDB0231434 and DDB0217453) encoding putative glutathione transferases, which were down-regulated significantly. Glutathione transferase catalyses the conjugation of reduced glutathione to ROS, thereby mediating detoxification (Kenneth T. Douglas, 2006). A possible explanation for the different expression pattern of genes encoding components of the antioxidant system is that *Dictyostelium* might mediate detoxification of ROS independent of glutathione during osmostress. Another possibility is that the up-regulation of glutathione S-transferase domain containing

protein is the important enzyme in this process while the other three putative glutathione transferase are not involved.

4.2.4 Contractile vacuole

The v-ATPase is a rotary molecular motor that uses hydrolysis of ATP to pump protons across membranes (Nelson and Harvey, 1999). Specific inhibition of v-ATPase by Concanamycin A increases the pH of endo-lysosomal vesicles and leads to formation of large intracellular vacuoles containing fluid phase. Therefore, it was suggested that v-ATPase functions to regulate membrane fusion (Temesvari et al., 1996). In *Dictyostelium,* the v-ATPase is primarily localized in membranes of the contractile vacuole, an osmoregulatory organelle. Mutant *Dictyostelium* cells with reduced v-ATPase levels showed defects in endocytic function and cytosolic pH regulation but did not manifest osmoregulatory defects (Liu et al., 2002). We found that all subunits of the vacuolar ATPase (v-ATPase) were down-regulated in a similar way (Figure 25). These results suggest that down-regulation of the v-ATPase is part of the cellular response to hyperosmolarity that actually might protect *Dictyostelium* cells from these adverse environmental conditions.

4.2.5 Development and osmostress response

Interestingly, we also found an enrichment of genes involved in developmental processes and fruiting body formation in all four clusters of figure 17 (Figure 17 and Table SI 1). There is a long and parallel history of the effects of osmotic pressure on vegetative cells and developing spores. The formation of dormant spores requires a high osmotic pressure exerted by the matrix between the spores, which consists largely of ammonium phosphate at a 100-200 mM concentration (Cotter et al., 1999). This leads to a raise in cAMP levels in the spore through the activation of adenylyl cyclase G (ACG) which functions as an intramolecular osmosensor (Alvarez-Curto et al., 2007; Virdy et al., 1999). The increase in intracellular cAMP in turn activates PKA which inhibits spore germination (Cotter et al., 2000; van Es et al., 1996).

Another intriguing parallel between hyperosmotically stressed vegetative cells and developing spores is the tyrosine phosphorylation of actin (Howard et al., 1993; Jungbluth et al., 1995; Kishi et al., 1998). The phosphorylation of actin during development is reduced in the *srfA*$^{-}$ mutant (Escalante et al., 2004b). *SrfA* encodes a MADS-box (**M**CM1, **A**RG80 from yeast, **D**eficient from Arabidopsis and **S**RF from

humans) transcription factor. It plays an important role in the development of *Dictyostelium*, as it is required for full maturation of spores (Escalante and Sastre, 1998). *SrfA* itself was slightly up-regulated upon sorbitol treatment (Table 4). In addition, several *srfA*-induced genes were highly up-regulated (Table 6). Therefore we reasoned that SrfA might be involved in the transcriptional response to hypertonicity. However, comparison of the *srfA*$^-$ mutant with wild type cells resulted in no significant transcriptional differences (Table 7). We therefore conclude that *srfA* is not involved in the transcriptional regulation to hypertonicity.

The enrichment of developmental genes in response to hyperosmotic stress is best explained if one assumes that the mechanisms which evolved to protect vegetative *Dictyostelium* cells from high osmolarity have been adapted for developmental processes.

4.3 STATc is a key regulator of the transcriptional response to hyperosmotic stress

We were interested in components of signal transduction pathway(s) that could get activated in response to sorbitol. We reasoned that signaling components might be among the early differentially regulated genes.

At 15 minutes post treatment only 38 genes were differentially regulated and 35 of these were up-regulated. Manual annotation revealed several interesting genes in this group (Table 6), among them STATc. STAT proteins are latent transcription factors that dimerise upon activation through tyrosine phosphorylation followed by translocation to the nucleus where they regulate the expression of target genes (Bromberg and Chen, 2001; Horvath, 2000). There are four different STAT proteins encoded in the *Dictyostelium* genome, however, no STATs are present in yeast (Williams et al., 2005). In the time course experiment with sorbitol-treated AX2 wild type cells we found STATa, b and c up-regulated, however, induction of STATc was most pronounced. Interestingly, when mammalian cells are subjected to osmotic or oxidative stress they activate JAK-STAT signaling pathways in addition to MAPK cascades (Bode et al., 1999; Carballo et al., 1999; Gatsios et al., 1998).

To learn more about the role of STATc in signal transduction upon hyperosmotic shock in *Dictyostelium* we made use of a STATc null mutant (Fukuzawa et al., 2001) and compared the expression profiles of AX2 wt treated

versus untreated with STATc knock-out treated versus untreated and RIC (random integrant cells) treated versus STATc knock-out treated cells. Our results show that STATc regulates the expression of approximately 20% of the more than 700 genes that were common between two or three of the above comparisons. In particular, we found that STATc dominated the osmostress-dependent expression of genes in clusters 4 and 7 (Figure 31). For most of these genes, including STATc itself, we observed the first transcriptional changes already 15 or 30 minutes post treatment. This period of time appears not to be sufficient for de novo expression of a STATc-dependent transcription factor that would then differentially regulate the observed target genes. Therefore, we assume that STATc directly regulates the expression of these genes.

Another intriguing result was that STATc was responsible for the up- as well as down-regulation of target genes. This result can be explained if we assume that STATc acts together with a transcriptional activator for the up-regulation and/or a transcriptional repressor for the down-regulation of target genes (Figure 39). The nature of the putative transcriptional cofactor(s) is currently unknown.

GO annotation of cluster 4 and 7 genes showed that STATc is responsible for the coordinated regulation of genes in distinct functional categories. Among the STATc-dependent genes we found an enrichment of genes involved in proton transport (due to v-ATPase subunits), actin-filament based processes, in the response to osmotic stress and culmination during fruiting body formation (Figure 32). During development STATc is activated by DIF, which induces the differentiation of prestalk O cells (Fukuzawa et al., 2001; Thompson and Kay, 2000). Previous work suggested a clear separation of the STATc target genes in development and stress (Araki et al., 2003). In contrast, our GO results (Figure 30) show the enrichment of genes in the categories “development” and “culmination during fruiting body formation”.

STATc was also responsible for the differential regulation of a number of metabolic enzymes and transporter genes, indicating that STATc could regulate the accumulation of some organic solutes. Araki et al. found a small difference in cell viability of wild type cells and the STATc mutant ($84 \pm 17\%$ and $59 \pm 21\%$, respectively) upon treatment with sorbitol. However, they claimed that the STATc mutant is not abnormally sensitive to hyperosmotic stress and attributed the

difference to experimental variance (Araki et al., 2003). Further experiments should clarify this issue.

In order to further investigate the STATc signaling pathway, we knocked out two possible candidates of STATc kinases, PkyA and DDB0231199. If either of them is the STATc tyrosine kinase, we would expect that STATc signaling upon osmostress is abolished. Experiments in this direction are underway.

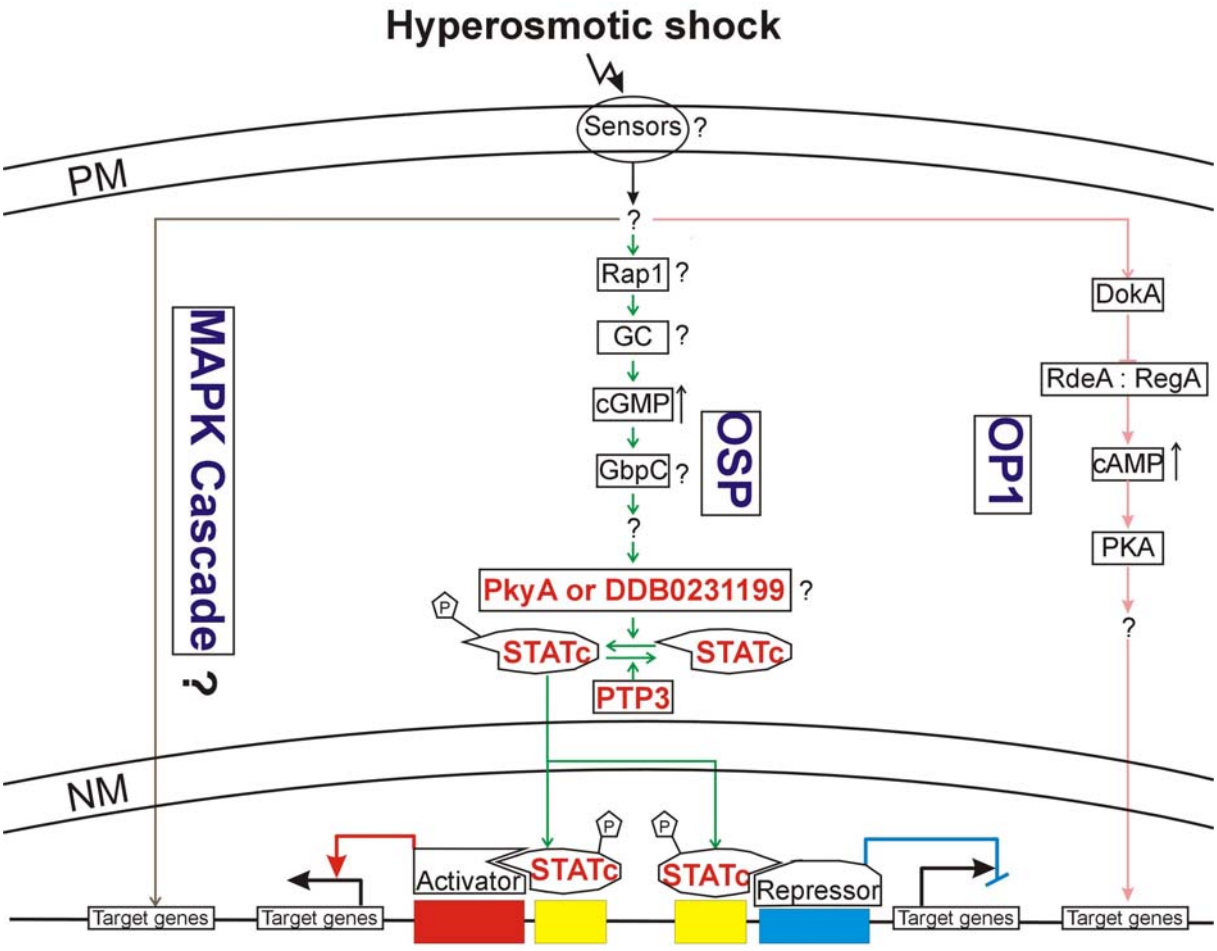

Figure 39 The model depicts known and putative signal transduction pathways that might get activated in *Dictyostelium* cells in response to hypertonicity. In contrast to yeast no osmosensors are so far known in *Dictyostelium*. The OP1 (osmostress-dependent pathway 1) pathway is under control of the hybrid histidine kinase DokA and leads to elevated cAMP levels thereby activating protein kinase A (PKA) (Schuster et al., 1996). The cGMP pathway, which we named OSP (osmostress-dependent STATc pathway), leads to the activation and nuclear translocation of STATc (Araki et al., 2003). Other components of this pathway are probably the small GTPase Rap1, a guanylate cyclase (GC), a cGMP binding protein (GbpC), PkyA or DDB0231199 and the tyrosine phosphatase PTP3. Based on differential regulation of putative MAPK components we propose a third signaling branch, which might be under the control of a MAPK cascade, similar to yeast and mammals. Genes that were up-regulated in the experiments are labelled in red. PM: plasma membrane; NM: nuclear membrane.

4.4 Signaling pathways in response to hypertonicity

In yeast the HOG signaling pathway is responsible for the adaptation of the cells to high osmolarity. It can be activated by either of two upstream pathways, the SHO1 and the SLN1 pathway, which converge on Pbs2, a MAPKK and scaffolding protein that brings together the other components of the MAPK cascade (de Nadal et al., 2002). SHO1 and SLN1 are putative yeast osmosensors and there is possibly a third one, Msb2 (Maeda et al., 1995; Maeda et al., 1994; O´Rourke and Herskowitz, 2002). Microarray analysis showed that Msb2 and SHO1 function in parallel and regulate identical gene sets in *hog*1 mutants (O´Rourke and Herskowitz, 2002). Investigation of the yeast transcriptional response at different osmolarities showed that different response pathways are triggered. The environmental stress response pathway is preferentially used during extreme osmotic stress, the SLN1 branch but not the Sho1 branch of the HOG pathway is used during modest osmotic stress while all three pathways contribute significantly to differential gene expression at intermediate osmolarities (Causton et al., 2001; O´Rourke and Herskowitz, 2004). Our results of the osmostress-dependent transcriptional regulation of STATc knock-out and wt cells are best explained if one assumes two or even three signaling pathways that get activated upon subjecting *Dictyostelium* cells to hyperosmotic conditions. This conclusion is also supported by previous findings, which pointed to the activation of two independent signaling branches in the *Dictyostelium* osmostress response. The hybrid histidine kinase DokA branch and downstream effectors and the cGMP branch, that might be under the control of Rap1 (Kang et al., 2002; Kuwayama et al., 1996; Ott et al., 2000; Schuster et al., 1996). STATc is either part of the cGMP branch or could define a third independent signaling branch. The activation and nuclear translocation of STATc upon addition of 8Br-cGMP argues for STATc being a component of the cGMP branch, however, osmotic stress induced STATc phosphorylation was still observed in a double mutant which lacked both known *Dictyostelium* guanylate cyclases (Araki et al., 2003). Putative regulators of STATc are protein tyrosine phophatase 3 (PTP3) and PkyA or DDB0231199, tyrosine kinase-like protein kinases with homology to the mammalian JAK kinase (Gamper et al., 1999; Kimmel, 2005). While none of the known components of the DokA pathway were differentially regulated in response to hypertonicity, we found that PTP3, PkyA and DDB0231199, like STATc, were up-regulated. Furthermore, we found in our list of differentially regulated genes several up-regulated protein kinases that could be

part of a MAPK cascade, thus raising the possibility that *Dictyostelium,* like yeast and mammals, also uses a MAPK cascade in response to osmotic stress. Figure 39 depicts known and putative components of the *Dictyostelium* osmotic response under the assumption of three parallel signaling pathways.

A comprehensive view on the osmotic stress response requires a detailed understanding of various cellular aspects such as signal sensing and transduction, control of transport processes and metabolism and differential regulation of transcription and translation. Future work should clarify the exact role of STATc and unravel further critical components of the signal chains that get activated in *Dictyostelium* under adverse osmotic conditions.

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Supplement

Table SI 1. List of enriched biological process, molecular function and cellular component GO terms of the clusters of the 2 hours time course of treatment with 200 mM sorbitol.

Cluster 1	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Biological process	GO:0050789	6	481	2,63	0,032	2	regulation of biological process
	GO:0009057	3	120	5,28	0,019	5	macromolecule catabolism
	GO:0043285	3	71	8,92	0,005	5	biopolymer catabolism
	GO:0030163	3	66	9,59	0,005	6	protein catabolism
	GO:0031154	2	12	35,18	0,002	6	culmination during fruiting body formation
	GO:0006508	5	188	5,61	0,002	7	proteolysis
	GO:0008154	2	35	12,06	0,012	6	actin polymerization and/or depolymerization
Cellular component	GO:0000502	2	28	15,95	0,015	4	proteasome complex (sensu Eukaryota)
	GO:0000267	3	70	9,57	0,015	3	cell fraction
	GO:0005625	2	33	13,54	0,015	4	soluble fraction

Cluster 2							
Biological process	GO:0000003	9	180	2,94	0,003	2	reproduction
	GO:0019953	4	59	3,99	0,034	3	sexual reproduction
	GO:0048232	3	34	5,19	0,034	5	male gamete generation
	GO:0007283	3	34	5,19	0,034	6	spermatogenesis
	GO:0009605	9	160	3,31	0,002	4	response to external stimulus
	GO:0042330	5	53	5,55	0,002	5	taxis
	GO:0009453	3	19	9,29	0,017	6	energy taxis
	GO:0042594	5	103	2,86	0,034	5	response to starvation
	GO:0009314	3	31	5,69	0,017	5	response to radiation
	GO:0009416	3	24	7,35	0,017	6	response to light stimulus
	GO:0042331	3	19	9,29	0,017	7	phototaxis
	GO:0042221	5	114	2,58	0,049	5	response to chemical stimulus
	GO:0006935	5	53	5,55	0,002	6	chemotaxis
	GO:0043170	36	1645	1,29	0,034	4	macromolecule metabolism
	GO:0019538	26	1075	1,42	0,034	5	protein metabolism
	GO:0043037	7	115	3,58	0,003	7	translation
	GO:0005975	9	174	3,04	0,003	5	carbohydrate metabolism
	GO:0044262	8	113	4,16	0,001	6	cellular carbohydrate metabolism
	GO:0000270	3	13	13,57	0,001	7	peptidoglycan metabolism
	GO:0009056	10	214	2,75	0,003	4	catabolism
	GO:0016052	3	41	4,30	0,034	6	carbohydrate catabolism
	GO:0051674	9	76	6,97	0	4	localization of cell
	GO:0015986	2	21	5,60	0,049	7	ATP synthesis coupled proton transport
	GO:0006754	2	21	5,60	0,049	7	ATP biosynthesis
	GO:0007275	13	420	1,82	0,034	2	development
	GO:0048608	5	96	3,06	0,034	3	reproductive structure development
	GO:0030582	5	96	3,06	0,034	4	fruiting body formation
	GO:0030587	5	96	3,06	0,034	5	fruiting body formation (sensu Dictyosteliida)
	GO:0016192	6	126	2,80	0,034	5	vesicle-mediated transport
	GO:0006897	5	81	3,63	0,017	6	endocytosis
	GO:0015985	2	21	5,60	0,049	7	energy coupled proton transport\, down electrochemical gradient
	GO:0042775	2	12	9,80	0,017	7	ATP synthesis coupled electron transport (sensu Eukaryota)
	GO:0006082	11	254	2,55	0,017	5	organic acid metabolism
	GO:0019752	11	254	2,55	0,017	6	carboxylic acid metabolism
	GO:0006090	2	15	7,84	0,034	7	pyruvate metabolism
	GO:0006091	11	265	2,44	0,017	5	generation of precursor metabolites and energy
	GO:0006119	4	34	6,92	0,003	6	oxidative phosphorylation
	GO:0015980	5	81	3,63	0,017	6	energy derivation by oxidation of organic compounds
	GO:0006092	4	58	4,06	0,017	7	main pathways of carbohydrate metabolism
	GO:0045333	3	22	8,02	0,017	7	cellular respiration
	GO:0042773	2	12	9,80	0,017	7	ATP synthesis coupled electron transport
	GO:0051186	7	116	3,55	0,017	5	cofactor metabolism
	GO:0006732	7	102	4,04	0,002	6	coenzyme metabolism
	GO:0006084	5	30	9,80	0	7	acetyl-CoA metabolism
	GO:0044260	27	1064	1,49	0,017	5	cellular macromolecule metabolism
	GO:0044267	25	1052	1,40	0,034	6	cellular protein metabolism
	GO:0006457	6	62	5,69	0,001	7	protein folding

Supplement

Cluster 2	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Biological process	GO:0008154	3	35	5,04	0,034	6	actin polymerization and/or depolymerization
	GO:0044248	9	197	2,69	0,017	5	cellular catabolism
	GO:0051187	3	19	9,29	0,017	6	cofactor catabolism
	GO:0009109	3	19	9,29	0,017	7	coenzyme catabolism
	GO:0044275	3	41	4,30	0,034	7	cellular carbohydrate catabolism
	GO:0016998	3	10	17,65	0,001	6	cell wall catabolism
	GO:0009059	11	327	1,98	0,034	6	macromolecule biosynthesis
	GO:0006412	11	303	2,14	0,017	7	protein biosynthesis
	GO:0043038	3	42	4,20	0,034	7	amino acid activation
	GO:0042127	2	14	8,40	0,034	5	regulation of cell proliferation
	GO:0051128	3	27	6,54	0,017	5	regulation of cell organization and biogenesis
	GO:0008064	2	19	6,19	0,049	6	regulation of actin polymerization and/or depolymerization
	GO:0030029	5	90	3,27	0,034	7	actin filament-based process
	GO:0051301	6	63	5,60	0,001	4	cell division
	GO:0000910	6	56	6,30	0	5	cytokinesis
	GO:0048515	3	26	6,79	0,017	4	spermatid differentiation
	GO:0048468	3	47	3,75	0,049	4	cell development
	GO:0007286	3	26	6,79	0,017	5	spermatid development
	GO:0007291	3	24	7,35	0,017	6	sperm individualization
	GO:0007610	9	100	5,29	0	2	behavior
	GO:0007626	9	92	5,75	0	3	locomotory behavior
	GO:0040011	9	83	6,38	0	4	locomotion
	GO:0006928	9	76	6,97	0	5	cell motility
Molecular function	GO:0008565	3	45	4,10	0,041	3	protein transporter activity
	GO:0015077	3	43	4,29	0,038	5	monovalent inorganic cation transporter activity
	GO:0015078	3	39	4,73	0,034	6	hydrogen ion transporter activity
	GO:0046933	2	20	6,14	0,045	7	hydrogen-transporting ATP synthase activity\, rotational mechanism
	GO:0005489	4	76	3,23	0,04	3	electron transporter activity
	GO:0005386	6	130	2,84	0,025	3	carrier activity
	GO:0015399	5	70	4,39	0,008	4	primary active transporter activity
	GO:0015405	4	57	4,31	0,02	5	P-P-bond-hydrolysis-driven transporter activity
	GO:0015450	2	10	12,29	0,017	6	protein translocase activity
	GO:0046961	2	19	6,47	0,041	7	hydrogen-transporting ATPase activity\, rotational mechanism
	GO:0008553	2	7	17,55	0,008	7	hydrogen-exporting ATPase activity\, phosphorylative mechanism
	GO:0005200	4	47	5,23	0,011	3	structural constituent of cytoskeleton
	GO:0016624	2	6	20,48	0,007	5	oxidoreductase activity\, acting on the aldehyde or oxo group of donors\, disulfide as acceptor
	GO:0016651	3	22	8,38	0,008	4	oxidoreductase activity\, acting on NADH or NADPH
	GO:0016886	3	48	3,84	0,046	4	ligase activity\, forming phosphoric ester bonds
	GO:0008452	3	43	4,29	0,038	5	RNA ligase activity
	GO:0016875	3	43	4,29	0,038	4	ligase activity\, forming carbon-oxygen bonds
	GO:0016876	3	43	4,29	0,038	5	ligase activity\, forming aminoacyl-tRNA and related compounds
	GO:0004812	3	43	4,29	0,038	6	tRNA ligase activity
	GO:0003796	2	11	11,17	0,02	6	lysozyme activity
	GO:0005515	17	397	2,63	0	3	protein binding
	GO:0051082	4	14	17,55	0	4	unfolded protein binding
	GO:0003779	4	79	3,11	0,043	5	actin binding
	GO:0051015	2	13	9,45	0,025	6	actin filament binding
Cellular component	GO:0043226	40	1409	1,29	0,022	2	organelle
	GO:0031252	4	44	4,14	0,022	3	leading edge
	GO:0005622	50	1844	1,23	0,022	3	intracellular
	GO:0031461	2	6	15,17	0,022	5	cullin-RING ubiquitin ligase complex
	GO:0019005	2	6	15,17	0,022	6	SCF ubiquitin ligase complex
	GO:0043229	40	1409	1,29	0,022	4	intracellular organelle
	GO:0005856	10	179	2,54	0,022	4	cytoskeleton
	GO:0015629	8	106	3,43	0,022	5	actin cytoskeleton
	GO:0005885	2	9	10,11	0,022	6	Arp2/3 protein complex
	GO:0005737	41	1043	1,79	0	4	cytoplasm
	GO:0045254	2	5	18,20	0,022	5	pyruvate dehydrogenase complex
	GO:0005739	13	261	2,27	0,022	5	mitochondrion
	GO:0016282	4	39	4,67	0,022	5	eukaryotic 43S preinitiation complex

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Cluster 2	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Cellular component	GO:0005852	2	6	15,17	0,022	6	eukaryotic translation initiation factor 3 complex
	GO:0005783	5	74	3,07	0,022	5	endoplasmic reticulum
	GO:0005938	5	41	5,55	0,022	5	cell cortex
	GO:0030863	2	10	9,10	0,022	6	cortical cytoskeleton
	GO:0030864	2	8	11,38	0,022	7	cortical actin cytoskeleton
	GO:0042995	4	44	4,14	0,022	3	cell projection
	GO:0031143	3	20	6,83	0,022	4	pseudopodium

Cluster 3							
Biological process	GO:0045324	2	4	19,93	0,004	7	late endosome to vacuole transport
	GO:0007275	22	420	2,09	0,001	2	development
	GO:0031154	3	12	9,97	0,003	6	culmination during fruiting body formation
	GO:0006979	4	20	7,97	0,001	6	response to oxidative stress
	GO:0000082	2	2	39,87	0,001	7	G1/S transition of mitotic cell cycle
	GO:0030154	10	123	3,24	0,001	3	cell differentiation
	GO:0030435	6	29	8,25	0	4	sporulation
Molecular function	GO:0005215	17	408	1,74	0,023	2	transporter activity
	GO:0015267	3	27	4,65	0,032	3	channel or pore class transporter activity
	GO:0015268	3	27	4,65	0,032	4	alpha-type channel activity
	GO:0005216	3	27	4,65	0,032	5	ion channel activity
	GO:0015662	3	31	4,05	0,043	6	ATPase activity\, coupled to transmembrane movement of ions\, phosphorylative mechanism
	GO:0016564	3	14	8,96	0,006	3	transcriptional repressor activity
	GO:0003774	3	29	4,33	0,038	2	motor activity
	GO:0000146	2	7	11,95	0,017	3	microfilament motor activity
	GO:0030234	6	101	2,48	0,039	2	enzyme regulator activity
	GO:0004857	3	13	9,65	0,005	3	enzyme inhibitor activity
	GO:0005095	2	4	20,91	0,005	4	GTPase inhibitor activity
	GO:0005096	2	12	6,97	0,039	4	GTPase activator activity
	GO:0005099	2	3	27,89	0,003	5	Ras GTPase activator activity
	GO:0004674	11	242	1,90	0,035	7	protein serine/threonine kinase activity
	GO:0016769	3	15	8,37	0,008	4	transferase activity\, transferring nitrogenous groups
	GO:0008483	3	15	8,37	0,008	5	transaminase activity
	GO:0004175	6	98	2,56	0,035	5	endopeptidase activity
	GO:0005515	19	397	2,00	0,004	3	protein binding
	GO:0042802	5	10	20,91	0	4	protein self binding
	GO:0046983	5	25	8,37	0	4	protein dimerization activity
	GO:0042803	5	8	26,14	0	5	protein homodimerization activity
	GO:0005522	2	9	9,30	0,027	4	profilin binding
	GO:0051219	3	9	13,94	0,002	4	phosphoprotein binding
	GO:0019899	3	30	4,18	0,04	4	enzyme binding
	GO:0051020	3	22	5,70	0,022	5	GTPase binding
	GO:0031267	3	22	5,70	0,022	6	small GTPase binding
	GO:0017048	3	15	8,37	0,008	7	Rho GTPase binding
	GO:0008092	6	87	2,88	0,025	4	cytoskeletal protein binding
	GO:0003779	6	79	3,18	0,017	5	actin binding
	GO:0003785	2	13	6,44	0,043	6	actin monomer binding

Cluster 4	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Biological process	GO:0007582	171	3393	1,05	0,003	2	physiological process
	GO:0009266	3	9	6,91	0,022	5	response to temperature stimulus
	GO:0009408	3	8	7,78	0,022	6	response to heat
	GO:0008152	154	2631	1,21	0	3	metabolism
	GO:0044238	135	2231	1,25	0	4	primary metabolism
	GO:0006629	23	205	2,33	0	5	lipid metabolism
	GO:0006807	18	178	2,10	0,002	4	nitrogen compound metabolism
	GO:0005975	17	174	2,03	0,004	5	carbohydrate metabolism
	GO:0044262	15	113	2,75	0	6	cellular carbohydrate metabolism
	GO:0009056	21	214	2,04	0,002	4	catabolism
	GO:0016052	8	41	4,05	0,001	6	carbohydrate catabolism
	GO:0000272	2	5	8,30	0,027	6	polysaccharide catabolism
	GO:0009058	72	598	2,50	0	4	biosynthesis
	GO:0042592	6	24	5,18	0,002	3	homeostasis
	GO:0050801	3	14	4,44	0,03	4	ion homeostasis
	GO:0006885	2	6	6,91	0,048	5	regulation of pH
	GO:0051453	2	5	8,30	0,027	6	regulation of cellular pH
	GO:0045851	2	4	10,37	0,027	6	pH reduction

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Cluster 4	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Biological process	GO:0051452	2	4	10,37	0,027	7	cellular pH reduction
	GO:0042593	3	5	12,44	0,002	4	glucose homeostasis
	GO:0031155	3	9	6,91	0,022	4	regulation of fruiting body formation
	GO:0031156	3	9	6,91	0,022	5	regulation of fruiting body formation (sensu Dictyosteliida)
	GO:0031157	3	8	7,78	0,022	6	regulation of aggregate size
	GO:0031158	2	3	13,83	0,022	7	negative regulation of aggregate size
	GO:0002119	3	17	3,66	0,048	5	larval development (sensu Nematoda)
	GO:0009987	165	3240	1,06	0,027	2	cellular process
	GO:0050875	162	3072	1,09	0,002	3	cellular physiological process
	GO:0006811	13	118	2,28	0,004	5	ion transport
	GO:0006812	12	102	2,44	0,003	6	cation transport
	GO:0015672	9	51	3,66	0,001	7	monovalent inorganic cation transport
	GO:0030705	3	9	6,91	0,022	6	cytoskeleton-dependent intracellular transport
	GO:0007018	3	8	7,78	0,022	7	microtubule-based movement
	GO:0006818	9	31	6,02	0	5	hydrogen transport
	GO:0015992	9	31	6,02	0	6	proton transport
	GO:0015985	9	21	8,89	0	7	energy coupled proton transport\, down electrochemical gradient
	GO:0044237	149	2376	1,30	0	4	cellular metabolism
	GO:0006766	4	25	3,32	0,03	5	vitamin metabolism
	GO:0006767	4	25	3,32	0,03	6	water-soluble vitamin metabolism
	GO:0006082	24	254	1,96	0,002	5	organic acid metabolism
	GO:0019752	24	254	1,96	0,002	6	carboxylic acid metabolism
	GO:0006730	3	7	8,89	0,003	5	one-carbon compound metabolism
	GO:0009117	24	94	5,30	0	6	nucleotide metabolism
	GO:0009259	16	45	7,37	0	7	ribonucleotide metabolism
	GO:0006220	4	11	7,54	0,002	7	pyrimidine nucleotide metabolism
	GO:0019362	3	17	3,66	0,048	7	pyridine nucleotide metabolism
	GO:0006163	16	43	7,72	0	7	purine nucleotide metabolism
	GO:0009141	12	32	7,78	0	7	nucleoside triphosphate metabolism
	GO:0009123	5	16	6,48	0,002	7	nucleoside monophosphate metabolism
	GO:0046483	9	51	3,66	0,001	5	heterocycle metabolism
	GO:0006091	35	265	2,74	0	5	generation of precursor metabolites and energy
	GO:0006119	10	34	6,10	0	6	oxidative phosphorylation
	GO:0015986	9	21	8,89	0	7	ATP synthesis coupled proton transport
	GO:0015980	14	81	3,58	0	6	energy derivation by oxidation of organic compounds
	GO:0006092	12	58	4,29	0	7	main pathways of carbohydrate metabolism
	GO:0051186	20	116	3,58	0	5	cofactor metabolism
	GO:0006732	19	102	3,86	0	6	coenzyme metabolism
	GO:0006752	11	37	6,17	0	7	group transfer coenzyme metabolism
	GO:0046034	10	22	9,43	0	6	ATP metabolism
	GO:0006754	9	21	8,89	0	7	ATP biosynthesis
	GO:0051258	5	32	3,24	0,027	7	protein polymerization
	GO:0006457	7	62	2,34	0,03	7	protein folding
	GO:0044247	2	5	8,30	0,027	7	cellular polysaccharide catabolism
	GO:0044255	22	175	2,61	0	5	cellular lipid metabolism
	GO:0008202	6	24	5,18	0,002	6	steroid metabolism
	GO:0006643	8	65	2,55	0,027	6	membrane lipid metabolism
	GO:0006665	4	26	3,19	0,048	7	sphingolipid metabolism
	GO:0008610	11	108	2,11	0,027	6	lipid biosynthesis
	GO:0006694	4	14	5,93	0,004	7	steroid biosynthesis
	GO:0006636	2	6	6,91	0,03	7	fatty acid desaturation
	GO:0044248	21	197	2,21	0	5	cellular catabolism
	GO:0044270	5	41	2,53	0,048	6	nitrogen compound catabolism
	GO:0044265	10	105	1,98	0,03	6	cellular macromolecule catabolism
	GO:0044275	8	41	4,05	0,001	7	cellular carbohydrate catabolism
	GO:0044249	65	536	2,52	0	5	cellular biosynthesis
	GO:0009165	19	66	5,97	0	6	nucleotide biosynthesis
	GO:0009260	15	43	7,23	0	7	ribonucleotide biosynthesis
	GO:0006221	4	10	8,30	0,002	7	pyrimidine nucleotide biosynthesis
	GO:0006164	15	41	7,59	0	7	purine nucleotide biosynthesis
	GO:0009142	11	30	7,60	0	7	nucleoside triphosphate biosynthesis
	GO:0009124	5	14	7,41	0	7	nucleoside monophosphate biosynthesis
	GO:0019856	4	5	16,59	0	7	pyrimidine base biosynthesis

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Cluster 4	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Biological process	GO:0009113	2	3	13,83	0,022	7	purine base biosynthesis
	GO:0009059	30	327	1,90	0	6	macromolecule biosynthesis
	GO:0006412	29	303	1,99	0	7	protein biosynthesis
	GO:0051188	12	62	4,01	0	6	cofactor biosynthesis
	GO:0009108	11	49	4,66	0	7	coenzyme biosynthesis
	GO:0006725	11	61	3,74	0	5	aromatic compound metabolism
	GO:0042558	2	4	10,37	0,027	6	pteridine and derivative metabolism
	GO:0009112	7	22	6,60	0	6	nucleobase metabolism
	GO:0006206	4	9	9,22	0,001	7	pyrimidine base metabolism
	GO:0006144	3	13	4,79	0,027	7	purine base metabolism
	GO:0046112	6	8	15,55	0	7	nucleobase biosynthesis
	GO:0019438	2	5	8,30	0,027	6	aromatic compound biosynthesis
	GO:0042559	2	4	10,37	0,027	7	pteridine and derivative biosynthesis
	GO:0006519	19	163	2,42	0	5	amino acid and derivative metabolism
	GO:0006575	5	30	3,46	0,027	6	amino acid derivative metabolism
	GO:0042398	2	5	8,30	0,027	7	amino acid derivative biosynthesis
	GO:0009308	17	168	2,10	0,003	5	amine metabolism
	GO:0006520	14	138	2,10	0,022	6	amino acid metabolism
	GO:0009310	5	41	2,53	0,048	6	amine catabolism
	GO:0006066	15	85	3,66	0	5	alcohol metabolism
	GO:0016125	5	18	5,76	0,002	6	sterol metabolism
	GO:0016126	4	12	6,91	0,002	7	sterol biosynthesis
	GO:0008204	3	8	7,78	0,022	7	ergosterol metabolism
	GO:0005996	8	45	3,69	0,002	6	monosaccharide metabolism
	GO:0019318	8	44	3,77	0,002	7	hexose metabolism
	GO:0046164	6	23	5,41	0,001	6	alcohol catabolism
	GO:0046365	6	22	5,66	0	7	monosaccharide catabolism
	GO:0019725	3	16	3,89	0,048	4	cell homeostasis
	GO:0006873	3	13	4,79	0,027	5	cell ion homeostasis
	GO:0030003	3	13	4,79	0,027	6	cation homeostasis
	GO:0030004	2	6	6,91	0,048	7	monovalent inorganic cation homeostasis

Molecular function	GO:0005215	29	408	1,59	0,008	2	transporter activity
	GO:0015075	15	147	2,28	0,002	3	ion transporter activity
	GO:0008324	13	121	2,40	0,003	4	cation transporter activity
	GO:0015077	11	43	5,72	0	5	monovalent inorganic cation transporter activity
	GO:0015078	11	39	6,30	0	6	hydrogen ion transporter activity
	GO:0046933	9	20	10,05	0	7	hydrogen-transporting ATP synthase activity\, rotational mechanism
	GO:0005386	17	130	2,92	0	3	carrier activity
	GO:0015399	11	70	3,51	0	4	primary active transporter activity
	GO:0015405	10	57	3,92	0	5	P-P-bond-hydrolysis-driven transporter activity
	GO:0043492	11	117	2,10	0,016	3	ATPase activity\, coupled to movement of substances
	GO:0019829	8	27	6,62	0	6	cation-transporting ATPase activity
	GO:0046961	8	19	9,41	0	7	hydrogen-transporting ATPase activity\, rotational mechanism
	GO:0008553	3	7	9,57	0,003	7	hydrogen-exporting ATPase activity\, phosphorylative mechanism
	GO:0045182	7	55	2,84	0,012	2	translation regulator activity
	GO:0005198	20	177	2,52	0	2	structural molecule activity
	GO:0003735	17	95	4,00	0	3	structural constituent of ribosome
	GO:0003824	117	2325	1,12	0,026	2	catalytic activity
	GO:0004372	2	6	7,45	0,028	6	glycine hydroxymethyltransferase activity
	GO:0016769	3	15	4,47	0,028	4	transferase activity\, transferring nitrogenous groups
	GO:0008483	3	15	4,47	0,028	5	transaminase activity
	GO:0016765	6	34	3,94	0,004	4	transferase activity\, transferring alkyl or aryl (other than methyl) groups
	GO:0046912	3	10	6,70	0,009	5	transferase activity\, transferring acyl groups\, acyl groups converted into alkyl on transfer
	GO:0004108	2	5	8,94	0,019	6	citrate (Si)-synthase activity
	GO:0003878	2	2	22,34	0,002	6	ATP citrate synthase activity
	GO:0016491	37	377	2,19	0	3	oxidoreductase activity
	GO:0016639	2	2	22,34	0,002	5	oxidoreductase activity\, acting on the CH-NH2 group of donors\, NAD or NADP as acceptor
	GO:0016645	3	11	6,09	0,012	4	oxidoreductase activity\, acting on the CH-NH group of donors
	GO:0016646	3	10	6,70	0,009	5	oxidoreductase activity\, acting on the CH-NH group of donors\, NAD or NADP as acceptor

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Cluster 4	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Molecular function	GO:0016627	4	22	4,06	0,016	4	oxidoreductase activity\, acting on the CH-CH group of donors
	GO:0016903	5	22	5,08	0,003	4	oxidoreductase activity\, acting on the aldehyde or oxo group of donors
	GO:0016620	4	16	5,59	0,005	5	oxidoreductase activity\, acting on the aldehyde or oxo group of donors\, NAD or NADP as acceptor
	GO:0016667	2	6	7,45	0,028	4	oxidoreductase activity\, acting on sulfur group of donors
	GO:0016668	2	4	11,17	0,012	5	oxidoreductase activity\, acting on sulfur group of donors\, NAD or NADP as acceptor
	GO:0016616	7	73	2,14	0,045	5	oxidoreductase activity\, acting on the CH-OH group of donors\, NAD or NADP as acceptor
	GO:0016829	11	82	3,00	0,001	3	lyase activity
	GO:0016835	4	26	3,44	0,028	4	carbon-oxygen lyase activity
	GO:0016830	7	29	5,39	0	4	carbon-carbon lyase activity
	GO:0016831	5	22	5,08	0,003	5	carboxy-lyase activity
	GO:0004611	2	4	11,17	0,012	6	phosphoenolpyruvate carboxykinase activity
	GO:0004612	2	2	22,34	0,002	7	phosphoenolpyruvate carboxykinase (ATP) activity
	GO:0016863	2	3	14,89	0,007	5	intramolecular oxidoreductase activity\, transposing C=C bonds
	GO:0008234	6	57	2,35	0,042	5	cysteine-type peptidase activity
	GO:0019238	2	4	11,17	0,012	6	cyclohydrolase activity
	GO:0016812	2	5	8,94	0,019	5	hydrolase activity\, acting on carbon-nitrogen (but not peptide) bonds\, in cyclic amides
	GO:0016820	11	119	2,07	0,018	5	hydrolase activity\, acting on acid anhydrides\, catalyzing transmembrane movement of substances
	GO:0042626	11	117	2,10	0,016	6	ATPase activity\, coupled to transmembrane movement of substances
	GO:0042625	10	49	4,56	0	7	ATPase activity\, coupled to transmembrane movement of ions
	GO:0008135	7	55	2,84	0,012	4	translation factor activity\, nucleic acid binding
Cellular component	GO:0000221	2	3	12,04	0,018	7	hydrogen-transporting ATPase V1 domain
	GO:0016469	10	23	7,85	0	6	proton-transporting two-sector ATPase complex
	GO:0005622	112	1844	1,10	0,044	3	intracellular
	GO:0030529	21	168	2,26	0,001	4	ribonucleoprotein complex
	GO:0005737	92	1043	1,59	0	4	cytoplasm
	GO:0005773	8	52	2,78	0,018	5	vacuole
	GO:0005774	3	8	6,77	0,018	6	vacuolar membrane
	GO:0016471	3	5	10,84	0,004	7	hydrogen-translocating V-type ATPase complex
	GO:0005840	18	113	2,88	0	5	ribosome
	GO:0015934	4	20	3,61	0,04	6	large ribosomal subunit
	GO:0005739	28	261	1,94	0,001	5	mitochondrion
	GO:0005829	17	141	2,18	0,004	5	cytosol

Table SI 2. List of enriched biological process, molecular function and cellular component GO terms of clusters 4 and 7 of STATc-dependent genes.

Cluster 4	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Biological process	GO:0009058	9	598	2,84	0,007	4	biosynthesis
	GO:0006811	5	118	8,00	0	5	ion transport
	GO:0006812	4	102	7,41	0,007	6	cation transport
	GO:0015672	4	51	14,81	0	7	monovalent inorganic cation transport
	GO:0006818	4	31	24,37	0	5	hydrogen transport
	GO:0015992	4	31	24,37	0	6	proton transport
	GO:0015985	3	21	26,98	0	7	energy coupled proton transport\, down electrochemical gradient
	GO:0009117	3	94	6,03	0,023	6	nucleotide metabolism
	GO:0009259	3	45	12,59	0,006	7	ribonucleotide metabolism
	GO:0009150	3	41	13,82	0,001	7	purine ribonucleotide metabolism
	GO:0009141	3	32	17,70	0,001	7	nucleoside triphosphate metabolism
	GO:0006119	3	34	16,66	0,001	6	oxidative phosphorylation
	GO:0015986	3	21	26,98	0	7	ATP synthesis coupled proton transport
	GO:0051186	5	116	8,14	0	5	cofactor metabolism
	GO:0006732	5	102	9,26	0	6	coenzyme metabolism
	GO:0006752	3	37	15,31	0,001	7	group transfer coenzyme metabolism
	GO:0046034	3	22	25,75	0	6	ATP metabolism
	GO:0006754	3	21	26,98	0	7	ATP biosynthesis
	GO:0044249	9	536	3,17	0,001	5	cellular biosynthesis
	GO:0009165	3	66	8,58	0,014	6	nucleotide biosynthesis
	GO:0009260	3	43	13,18	0,006	7	ribonucleotide biosynthesis
	GO:0006164	3	41	13,82	0,001	7	purine nucleotide biosynthesis
	GO:0009142	3	30	18,88	0,001	7	nucleoside triphosphate biosynthesis
	GO:0051188	4	62	12,18	0	6	cofactor biosynthesis
	GO:0009108	4	49	15,42	0	7	coenzyme biosynthesis
Molecular function	GO:0015075	8	147	5,57	0,002	3	ion transporter activity
	GO:0008324	5	121	5,42	0,006	4	cation transporter activity
	GO:0015077	4	43	15,24	0	5	monovalent inorganic cation transporter activity
	GO:0015078	4	39	16,80	0	6	hydrogen ion transporter activity
	GO:0046933	3	20	24,57	0	7	hydrogen-transporting ATP synthase activity\, rotational mechanism
	GO:0005386	7	130	7,56	0	3	carrier activity
	GO:0015399	4	70	9,36	0,001	4	primary active transporter activity
	GO:0015405	4	57	11,50	0	5	P-P-bond-hydrolysis-driven transporter activity
	GO:0043492	5	117	7,00	0,001	3	ATPase activity\, coupled to movement of substances
	GO:0019829	3	27	18,20	0,001	6	cation-transporting ATPase activity
	GO:0046961	3	19	25,87	0	7	hydrogen-transporting ATPase activity\, rotational mechanism
	GO:0016820	5	119	6,88	0,001	5	hydrolase activity\, acting on acid anhydrides\, catalyzing transmembrane movement of substances
	GO:0042626	5	117	7,00	0,001	6	ATPase activity\, coupled to transmembrane movement of substances
	GO:0042625	4	49	13,37	0	7	ATPase activity\, coupled to transmembrane movement of ions
Cellular component	GO:0016282	2	39	7,00	0,032	3	eukaryotic 43S preinitiation complex
	GO:0005615	3	42	9,75	0,01	3	extracellular space
	GO:0016469	4	23	23,74	0	6	proton-transporting two-sector ATPase complex
	GO:0015935	2	31	8,81	0,021	6	small ribosomal subunit
	GO:0016283	2	30	9,10	0,021	5	eukaryotic 48S initiation complex
	GO:0005843	2	30	9,10	0,021	7	cytosolic small ribosomal subunit (sensu Eukaryota)

Supplement

Cluster 7	GO_ID	List	Total	Enrichment	P_value	GO_level	GO_Annotation
Biological process	GO:0006970	2	29	6,87	0,044	5	response to osmotic stress
	GO:0006807	5	178	2,80	0,044	4	nitrogen compound metabolism
	GO:0030048	2	6	33,22	0,002	5	actin filament-based movement
	GO:0042592	2	24	8,31	0,024	3	homeostasis
	GO:0050801	2	14	14,24	0,011	4	ion homeostasis
	GO:0031154	3	12	24,92	0	6	culmination during fruiting body formation
	GO:0006818	2	31	6,43	0,044	5	hydrogen transport
	GO:0015992	2	31	6,43	0,044	6	proton transport
	GO:0006800	2	28	7,12	0,044	5	oxygen and reactive oxygen species metabolism
	GO:0006979	2	20	9,97	0,018	6	response to oxidative stress
	GO:0009308	5	168	2,97	0,044	5	amine metabolism
	GO:0006520	4	138	2,89	0,049	6	amino acid metabolism
	GO:0016043	10	517	1,93	0,044	4	cell organization and biogenesis
	GO:0006996	7	315	2,21	0,044	5	organelle organization and biogenesis
	GO:0007032	2	14	14,24	0,011	6	endosome organization and biogenesis
	GO:0007010	5	154	3,24	0,018	6	cytoskeleton organization and biogenesis
	GO:0030029	5	90	5,54	0,003	7	actin filament-based process
	GO:0019725	2	16	12,46	0,012	4	cell homeostasis
	GO:0006873	2	13	15,33	0,011	5	cell ion homeostasis
	GO:0030003	2	13	15,33	0,011	6	cation homeostasis
	GO:0030004	2	6	33,22	0,002	7	monovalent inorganic cation homeostasis
	GO:0051301	3	63	4,75	0,044	4	cell division
	GO:0000910	3	56	5,34	0,018	5	cytokinesis
Molecular function	GO:0016564	2	14	15,18	0,01	3	transcriptional repressor activity
	GO:0000146	2	7	30,36	0,003	3	microfilament motor activity
	GO:0016769	2	15	14,17	0,01	4	transferase activity\, transferring nitrogenous groups
	GO:0008483	2	15	14,17	0,01	5	transaminase activity
	GO:0008233	5	174	3,05	0,024	4	peptidase activity
	GO:0008237	4	36	11,81	0	5	metallopeptidase activity
	GO:0008238	3	31	10,28	0,004	5	exopeptidase activity
	GO:0008235	3	14	22,77	0	6	metalloexopeptidase activity
	GO:0016160	2	3	70,85	0	6	amylase activity
	GO:0004556	2	3	70,85	0	7	alpha-amylase activity
	GO:0042802	4	10	42,51	0	4	protein self binding
	GO:0046983	4	25	17,00	0	4	protein dimerization activity
	GO:0042803	4	8	53,14	0	5	protein homodimerization activity
	GO:0051219	2	9	23,62	0,004	4	phosphoprotein binding
	GO:0008092	5	87	6,11	0,002	4	cytoskeletal protein binding
	GO:0003779	5	79	6,73	0,001	5	actin binding
	GO:0051015	2	13	16,35	0,009	6	actin filament binding
Cellular component	GO:0015629	5	106	5,52	0,002	5	actin cytoskeleton

Summary

Dictyostelium discoideum is frequently subjected to environmental changes in its natural habitat, the forest soil. In order to survive, the organism had to develop effective mechanisms to sense and respond to such changes. When cells are faced with a hypertonic environment a complex response is triggered. It starts with signal sensing and transduction and leads to changes in cell shape, the cytoskeleton, transport processes, metabolism and gene expression. Certain aspects of the *Dictyostelium* osmotic stress response have been elucidated, however, no comprehensive picture was available up to now.

To better understand the *D. discoideum* response to hyperosmotic conditions, we applied different methods. We first confirmed that actin is tyrosine phosphorylated, the F-actin cytoskeleton is redistributed, the cell volume is decreased considerably and cell viability is slightly diminished upon sorbitol treatment. Next, gene expression profiling using DNA microarrays was performed. Treatment of *Dictyostelium* cells with 200 mM sorbitol for 1 hour led to dramatic transcriptional changes, of which some were validated by real time PCR or Northern Blot. The transcriptional profile of cells treated during a 2-hour time course revealed a time-dependent induction or repression of 809 genes, more than 15% of the genes on the array, which peaked 45 to 60 minutes after the hyperosmotic shock. The differentially regulated genes were applied to cluster analysis and functional annotation using gene ontology (GO) terms. Two main responses appear to be the down-regulation of the metabolic machinery and the up-regulation of the stress response system, among them STATc (signal transducer and activator of transcription). Manual annotation revealed that many genes of the major metabolic pathways, including carbohydrate, amino acid and nucleotide metabolism were differentially regulated and could be responsible for the generation of osmolytes. We hypothesize that *Dictyostelium* uses a mixture of osmolytes to counteract the hyperosmotic stress. Interestingly we also found a number of differentially regulated genes that are involved in development. This is consistent with the notion that the cellular processes that protect amoebae from a hypertonic environment have been adapted for regulatory developmental process.

Gene expression profiling with the STATc mutant and appropriate controls showed that STATc is a key regulator of the transcriptional response to hyperosmotic shock. Approximately 20% of the differentially regulated genes that were common

between two or three experiments were dependent on the presence of STATc. Our results suggest that at least two signalling pathways are activated in *Dictyostelium* cells subjected to hypertonicity. STATc is responsible for the transcriptional changes of one of them.

Zusammenfassung

Dictyostelium discoideum ist in seiner natürlichen Umgebung, dem Waldboden, häufig von ökologischen Veränderungen betroffen. Zum Überleben mußte der Organismus daher effektive Mechanismen entwickeln, um solche Veränderungen wahrnehmen und darauf reagieren zu können. Wenn Zellen einer hypertonen Umgebung ausgesetzt werden, wird eine komplexe Antwort ausgelöst. Sie beginnt mit der Wahrnehmung und der Weitergabe des Signals und führt zu Veränderungen der Zellform, dem Zytoskelett, von Transportprozessen, dem Metabolismus und in der Genexpression. Bestimmte Aspekte der osmotischen Stressantwort von *Dictyostelium* wurden bereits aufgeklärt, aber bisher war keine umfassende Darstellung möglich.

Um die Antwort von *D. discoideum* auf hyperosmotische Bedingungen besser verstehen zu können, haben wir verschiedene Methoden angewandt. Wir konnten bestätigen, dass nach Sorbitolbehandlung Aktin am Tyrosin phosphoryliert, das F-Aktin Zytoskelett umverteilt, das Zellvolumen deutlich reduziert und die Lebensfähigkeit der Zellen etwas vermindert wird. Im nächsten Schritt wurde mittels DNA *Microarrays* ein Muster der Genexpression erstellt. Eine Behandlung der *Dictyostelium* Zellen mit 200 mM Sorbitol für eine Stunde bewirkte umfassende transkriptionelle Veränderungen, von denen einige durch "*real time* PCR" oder "*Northern Blot*" bestätigt wurden. Das transkriptionelle Profil von Zellen, die für zwei Stunden behandelt wurden, zeigte eine zeitabhängige Induktion oder Repression von 809 Genen, mehr als 15% der Gene auf dem "*Array*". Am meisten Gene waren 45 bis 60 Minuten nach dem hyperosmotischen Schock differentiell reguliert. Die 809 Gene wurden zur Clusteranalyse und funktionellen Analyse mittels "*Gene Ontology*" *(GO)* herangezogen. Zwei wesentliche Antworten scheinen die Abregulierung der metabolischen Maschinerie und die Aufregulierung des Stressantwortsystems, darunter STATc (signal transducer and activator of transcription), zu sein. Die manuelle Annotation zeigte, dass viele Gene der wichtigsten Stoffwechselwege, einschließlich des Kohlenhydrat-, Aminosäuren- und Nukleotid-metabolismus differentiell reguliert werden und für die Synthese von Osmolyten verantwortlich sein könnten. Wir nehmen an, dass *Dictyostelium* mehrere Osmolyte verwendet um dem hyperosmotischen Stress entgegenzuwirken. Interessanterweise haben wir außerdem eine Anzahl differentiell regulierte Gene gefunden, die in die

Entwicklung involviert sind. Dies unterstützt die Hypothese, dass zelluläre Prozesse, die die Amöbe vor einer hypertonen Umgebung schützen, für regulatorische Entwicklungsprozesse angepasst worden sind.

Genexpressionsanalysen mit einer STATc Mutante und geeigneten Kontrollen zeigten, dass STATc ein Schlüsselregulator für die transkriptionelle Antwort auf hyperosmotischen Schock ist. Die differentielle Regulation von etwa 20% der Gene, die zwei oder drei der vergleichenden Experimente gemeinsam waren, war abhängig von der Anwesenheit von STATc. Unsere Ergebnisse lassen den Schluss zu, dass in *Dictyostelium* Zellen, die hypertonischen Bedingungen ausgesetzt werden, mindestens zwei Signalwege aktiviert werden. STATc ist verantwortlich für die transkriptionellen Änderungen in einem dieser Wege.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen und Abbildungen -, die anderen Werke im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht ist, sowie, dass ich eine Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Frau Prof. Dr. Angelika A. Noegel betreut worden.

Köln den

Jianbo Na

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Curriculum Vitae

Name:	Jianbo Na
Address:	Nikolausstr. 86 D-50937, Cologne, Germany
Date of birth:	26.12.1976
Nationality:	P. R. China

School studies:

1992-1995	The No. 1 High School of Kunming, Yunnan, China

University studies:

1995-1999	Bachelor of Science in Biochemistry, Shandong University, China
1999-2002	Master of Science in Human Genetics, Chinese Academy of Medical Sciences & Peking Union Medical College, China

Doctoral studies:

09/2003-11/2006	PhD fellowship sponsored by "International Graduate School in Genetics and Functional Genomics", University of Cologne
04/2004-06/2007	Supervisor: Prof. Dr. Angelika A. Noegel & PD. Dr. Ludwig Eichinger, Institute for Biochemistry I, Medical Faculty, University of Cologne, D-50931 Cologne, Germany

Lebenslauf

Name, Vorname:	Na, Jianbo
Addresse:	Nikolausstr. 86 D-50937 Köln, Deutschland
Geburtsdatum:	26.12.1976
Nationalität:	V. R. China
Schulausbildung:	
1992-1996	The No. 1 High School of Kunming, Yunnan, China
Universität Studium:	
1995-1999	Bachelor of Science in Biochemistry, Shandong University, China
1999-2002	Master of Science in Human Genetics, Chinese Academy of Medical Sciences & Peking Union Medical College, China
Promotionsstudium:	
09/2003-11/2006	Stipendiat bei der “International Graduate School in Genetics and Functional Genomics”, Universität zu Köln
04/2004-06/2007	Betreuer(in): Prof. Dr. Angelika A. Noegel & PD. Dr. Ludwig Eichinger, Institut für Biochemie I, Medizinische Fakultät, Universität zu Köln, D-50931 Köln, Deutschland