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Original Article

Global gene expression profile of *schizosaccharomyces pombe* cells to sodium fluoride

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Abstract: Objective: Fluoride is essential for human and animal health and at recommended dosage in the millimolar range. In the appropriate dosage range, fluoride is safe and effective for the treatment of caries, but excessive administration usually results in tissue damage. Fluoride's physiological functions and mechanisms of toxicity are complex and incompletely understood. The aim of this study was to investigate the physiological response of fission yeast to sodium fluoride (NaF). Methods: Using microarrays and real-time PCR, we assayed the transcriptional response of wild-type fission yeast cells to NaF. Results: We found that NaF caused differential expression of genes encoding transporters, stress response proteins, and transcription factors in *Schizosaccharomyces pombe*. We also identified catalytic activities, including those of dehydrogenase, kinase, phosphatase, and other enzymes. Moreover, analysis of previously identified environmental stress response genes revealed that many of the NaF-sensitive genes we identified are NaF-specific. Conclusions: These results suggest that *S. pombe* cells respond to sodium fluoride through global gene expression changes. These findings would improve the general understanding of the molecular function of NaF in cells.

Keywords: Gene expression, fission yeast, *S. pombe* cells, sodium fluoride, transporter

Introduction

Existing widely in soil, water, air, and the earth's crust [1-3], fluoride is essential for human and animal health. In a millimolar range, fluoride can affect diverse physiological functions [4], but excessive intake is toxic to a variety of organisms. It is commonly used for the treatment of oral diseases [5], such as dental caries, dentin allergies, and periodontitis. In an appropriate range, fluoride is safe and effective for the treatment of caries, but excessive administration usually results in tissue damage [5, 6]. Recently, it has been found that fluoride activates responsive riboswitches in eubacteria and archaea [7], and CLCF genes encoding F_/H_ antiporters in eubacteria, Fluc in bacteria, and FEX2 in eukaryotes [8]. The physiological functions of fluoride and the mechanisms of its toxicity are complex and incompletely understood.

Schizosaccharomyces pombe has been used as model organism to investigate basic biological processes [9-11], and a comprehensive database on its biology, 'PomBase', is available. This database provides literature curation, functional annotation, and access to genomic sequence and features as well as genome-wide data sets. Moreover, in recent years, various studies using microarray analysis have probed the global gene expression profiles and regulatory mechanisms of *S. pombe* in response to environmental stress.

In this study, we aimed to investigate the physiological response of *S. pombe* to sodium fluoride (NaF) in order to elucidate the mechanisms of NaF activity.

Materials and methods

Subjects of study

Wild type (WT) *S. pombe* 972h$^-$ was cultured in accordance with the methods previously described by Petersen et al [12]. NaF was used at concentration of 0 μM, 30 μM, 300 μM, 3 mM,

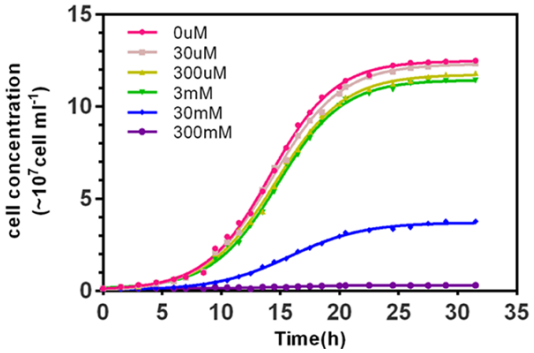

Figure 1. Growth curve of fission yeast cells in the presence of NaF. Wild type cells were grown in the presence of 0, 30 μM, 300 μM, 3 mM, 30 mM, and 300 mM NaF. Cell density was measured every 1.5 or 2 hours. The growth curve shows that addition of 300 mM NaF totally inhibits the growth of wild type cells.

30 mM, and 300 mM to treat yeast cells in log phase.

RNA extraction and purification

Following RNA extraction, reverse transcription was carried out with an appropriate kit (Takara). Real-time PCR reactions contained 250 nM of forward and reverse primers, 1 ml cDNA (5 ng), 10 ml 26SYBR-green Real time PCR Master Mix (SYBR Premix Ex TaqTM, Takara) in a total volume of 20 ml.

DNA microarray

Microarray experiments were done using the Affymetrix Yeast Genome 2.0 ArrayThis array is comprised of 5,021 probe sets for 5,031 genes identified in *S. pombe*. The principal stages of these experiments were RNA purification, cDNA probe preparation, hybridization, washing, scanning, and image analysis, with normalization and other processing of data as described in the Technical Manual of Affymetrix GeneChip_Expression Analysis. We performed three biological replicates for the microarray experiments. Data were analyzed as in Xu Jia, et al [13]. Genes identifi-ed by the microarray were an-notated with "gene ID" and "gene symbol" using the NetAffxTM Analysis Center. To effectively screen differentially expressed genes, the parameters of Significance Analysis of Microarrays (SAM) and fold change were used to pick out significant genes among the different groups, with the False Discovery Rate (FDR) set less than 0.1, and fold change greater than 1.5. The statistical significance (p value) of overlaps between two gene groups was evaluated by the Fisher Test. Functional classification was accomplished with the online software PANTHER [14, 15], and cluster 3.0 [16], and then visualized using TreeView, version 3 [15].

Real-time PCR

RNA used for Real-time PCR was from the same samples taken for microarray experiments. Real-time PCR was performed on an iQ5 Continuous Fluorescence Detector System (Bio-Rad). PCR reactions were done with 250 nM forward and reverse primers (Table S1), 1 μL cDNA (5 ng), and 10 μL 26 SYBR-green Real time PCR Master Mix (SYBR Premix Ex Taq™, TaKaRa) in a total volume of 20 μL. For all of our experiments, we performed at least three independent biological replicates, and for each of these, four technical replicates were performed.

Statistical analysis

Data are presented as mean ± SD unless otherwise indicated. The data were analyzed by Student's t-test or one-way analysis of variance (ANOVA) using Prism version 5 (GraphPad Software, Inc.). Values of $p < 0.05$ were considered statistically significant.

Results

Response of S. pombe cells to NaF at varying concentrations

Fluoride, as an antimicrobial agent, has been widely shown to improve oral health because of its toxic effects on bacteria or fungi, but its effects on *S. pombe* cells have not been studied. In this study, *S. pombe* cells were treated with NaF in different concentrations (0 μM, 30 μM, 300 μM, 3 mM, 30 mM and 300 mM) to determine genome-wide response to NaF. As shown in **Figure 1**, NaF could inhibit the growth of *S. pombe* cells in a dose-dependent manner; after being treated with varying concentrations of NaF, cells reached their maximal density after 24 h, with the exception that growth was completely inhibited in the presence of 300 mM NaF. No substantial changes in cell density were observed under the microscope until more than 0.5 h had elapsed. Because we wished to perform RNA analysis following two contrasting doses, on cells whose growth was only slightly affected, we chose to treat them with 30 μM or 300 μM NaF for 0.5 h.

Table 1. Genes whose expression level reached FDR < 0.1 and was changed by 1.5-fold or greater in response to 30 μM NaF treatment

Gene symbol	Regulation (30 μM)	Fold Change (30 μM)	Gene products
Stress response			
psu1	Down	0.5727	Cell wall protein Psu1, beta-glucosidase (predicted)
SPAC19B12.01	Down	0.6234	TPR repeat protein, TTC27 family
Pyp2	Up	1.8216	Tyrosine phosphatase Pyp2
Rrp9	Down	0.659	U3 snoRNP-associated protein Rrp9 (predicted)
Spacunk4.17	Up	1.7131	NAD binding dehydrogenase family protein
Spbc660.05	Up	3.0347	WW domain containing conserved fungal protein
Spbc725.03	Up	1.5227	Pyridoxamine 5'-phosphate oxidase (predicted)
Oca2	Up	2.0162	Serine/threonine protein kinase Oca2
Gut2	Up	1.9038	Glycerol-3-phosphate dehydrogenase Gut2 (predicted)
Set5	Down	0.5381	Histone lysine methyltransferase Set5 (predicted)
Cox3	Up	1.6161	Cytochrome c oxidase 3 (predicted)
Transporter activity (transmembrane transporter/iron transporter)			
Str3	Down	0.47	Siderophore-iron transporter Str3
Spac24h6.13	Down	0.6611	DUF221 family protein implicated in Golgi to plasma Membrane transport (predicted)
Gti1	Up	1.6527	Gluconate transmembrane transporter inducer
Spac869.03C	Up	1.5235	Urea transmembrane transporter (predicted)
Ght5	Up	3.5221	Hexose transmembrane transporter Ght5
Ght1	Up	2.2091	Hexose transmembrane transporter Ght1
Pfl8	Down	0.5928	Cell surface glycoprotein, flocculin Pfl8
Binding activity (DNA or RNA binding/transcription factor)			
Sfp1	Down	0.5406	Transcription factor Sfp1 (predicted)
SPAC2H10.01	Up	4.4577	Transcription factor, zf-fungal binuclear cluster type (predicted)
Cbf12	Up	1.6599	CBF1/Su(H)/LAG-1 family transcription factor Cbf12
Spac683.02C	Down	0.5505	Zf-CCHC type zinc finger protein (predicted)
Pof15	Up	2.5744	F-box protein (predicted)
Lsd90	Up	2.2633	Lsd90 protein
Catalytic Activity (dehydrogenase activity/kinase/phosphatase activity/others)			
Spac22a12.17C	Up	1.7317	Short chain dehydrogenase (predicted)
Isp7	Down	0.6444	2-OG-Fe(II) oxygenase superfamily protein
Srx1	Down	0.6121	Sulfiredoxin
Nar1	Down	0.5913	Iron hydrogenase Nar1 (predicted)
Spcc1739.08C	Up	1.8985	Short chain dehydrogenase (predicted)
Zwf2	Up	2.5825	Glucose-6-phosphate 1-dehydrogenase Zwf2 (predicted)
Shk2	Up	1.757	PAK-related kinase Shk2
Ark1	Down	0.627	Aurora-B kinase Ark1
Ctu2	Down	0.4546	Cytosolic thiouridylase subunit Ctu2
Alp41	Up	1.545	GTP-binding protein involved in beta-tubulin folding Alp41
Nep2	Up	2.7506	NEDD8 protease Nep2
Spbc336.02	Down	0.461	18S rRNA dimethylase (predicted)
Spbpb7e8.02	Up	1.5675	PSP1 family protein
Pma2	Down	0.4703	P-type proton ATPase, P3-type Pma2
Cell Cycle (meiosis/meiotic).			
Moa1	Up	2.6792	Meiotic kinetochore protein (Meikin) Moa1
Dic1	Down	0.6284	Meiotic dynein intermediate chain Dic1
Ribosome biogenesis			
Imp4	Down	0.6237	U3 snoRNP-associated protein Imp4(predicted)
Sda1	Down	0.6021	SDA1 family protein (predicted)
Bfr2	Down	0.545	Traub family protein involved in ribosome biogenesis (predicted)
Rrp1402	Down	0.6638	Ribosome biogenesis protein Rrp14 (predicted)
S.pombe Specific protein/Conserved Fungal Protein/other proteins			
SPAC17A2.10c	Up	4.3801	*Schizosaccharomyces pombe* specific protein

Spac23h3.15C	Up	2.567	Schizosaccharomyces specific protein
Mug114	Up	2.5958	*Schizosaccharomyces pombe* specific protein
Spapj695.01C	Down	0.5917	*S. pombe* specific UPF0321 family protein 3
Mug45	Down	0.6429	Schizosaccharomyces specific protein Mug45
Spac1952.04C	Up	1.6079	Conserved fungal protein
Spac4f10.17	Up	2.1388	Conserved fungal protein
Mug14	Up	1.685	Adducin,mug14
Wtf20	Up	1.927	Wtf element Wtf20
Wtf4	Up	1.8948	Wtf element Wtf4
Spac212.03	Up	3.0055	Hypothetical protein
Spap27g11.11C	Down	0.3035	Dubious
Spbcpt2r1.06C	Down	0.2018	Unassigned
Spbpb21e7.08	Up	1.625	Unassigned
Wtf3	Up	3.1686	Pseudogene
Wtf24	Up	1.6178	Pseudogene

Table 2. Genes whose expression level reached FDR < 0.1 and was changed by 1.5-fold or greater in response to 300 mM NaF treatment

Gene symbol	Regulation	Fold change	Gene products
Stress response			
Pyp2	1.8106	Up	tyrosine phosphatase Pyp2
Spacunk4.17	1.6513	Up	NAD binding dehydrogenase family protein
Spapb1a11.03	1.5249	Up	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase) (predicted)
Spbc1289.14	1.5515	Up	Adducin (predicted)
Spbc660.05	2.2035	Up	WW domain containing conserved fungal protein
Spbc725.03	1.515	Up	Pyridoxamine 5'-phosphate oxidase (predicted)
Oca2	2.172	Up	Serine/threonine protein kinase Oca2
Gut2	1.6952	Up	Glycerol-3-phosphate dehydrogenase Gut2 (predicted)
Spcc191.01	1.5286	Up	Schizosaccharomyces specific protein
Set5	0.6125	Down	Histone lysine methyltransferase Set5 (predicted)
Transporter activity (transmembrane transporter/iron transporter)			
Ght5	3.6205	Up	Hexose transmembrane transporter Ght5
Ght1	2.0812	Up	Hexose transmembrane transporter Ght1
Spcc794.04C	1.5674	Up	Amino acid transmembrane transporter (predicted)
Vta1	1.8167	Up	Vps20 associated protein Vta1 (predicted)
Gti1	1.8557	Up	Gluconate transmembrane transporter inducer Gti1
Str3	0.4742	Down	Siderophore-iron transporter Str3
Spac24h6.13	0.5957	Down	DUF221 family protein implicated in Golgi to plasma membrane transport (predicted)
Isp4	0.642	Down	OPT oligopeptide transmembrane transporter family Isp4
Pma2	0.6186	Down	P-type proton ATPase, P3-type Pma2
Pfl8	0.647	Down	Cell surface glycoprotein, flocculin pfl8
Binding activity (DNA or RNA binding/transcription factor)			
Sfp1	0.546	Down	Transcription factor sfp1 (predicted)
Spac2h10.01	4.7135	Up	Transcription factor, zf-fungal binuclear cluster type (predicted)
Cbf12	1.6674	Up	Cbf1/su(h)/lag-1 family transcription factor cbf12
Hsp3105	1.8328	Up	Thij domain protein
Spac683.02C	0.5714	Down	Zf-cchc type zinc finger protein (predicted)
Pof15	2.711	Up	F-box protein (predicted)
Spbc16c6.03C	1.5171	Up	Ribosome assembly protein (predicted)
Lsd90	1.7524	Up	Lsd90 protein
Catalytic Activity (dehydrogenase activity/kinase/phosphatase activity/others)			
Spac186.07C	0.3884	Down	Hydroxyacid dehydrogenase (predicted)
Isp7	0.6107	Down	2-Og-fe(ii) oxygenase superfamily protein
Srx1	0.6383	Down	Sulfiredoxin
Nar1	0.5974	Down	Iron hydrogenase nar1 (predicted)

SPCC1739.08c	1.8354	Up	Short chain dehydrogenase (predicted)
Zwf2	2.8519	Up	Glucose-6-phosphate 1-dehydrogenase zwf2 (predicted)
Shk2	1.792	Up	Pak-related kinase shk2
Pik1	1.8212	Up	1-Phosphatidylinositol 4-kinase pik1
Fip1	0.6605	Down	Iron permease fip1
SPAC869.04	5.6954	Up	Formamidase-like protein (predicted)
Ctu2	2.1257	Up	Cytosolic thiouridylase subunit ctu2
SPBC2G2.17c	1.6601	Up	Beta-glucosidase psu2 (predicted)
Nep2	3.1579	Up	Nedd8 protease nep2
SPBC336.02	0.4869	Down	18S rrna dimethylase (predicted)
SPBPB7E8.02	1.6131	Up	Psp1 family protein
Cell Cycle (meiosis/meiotic)			
Moa1	2.7484	Up	Meiotic kinetochore protein (meikin) moa1
Mei3	3.3802	Up	Meiosis inducing protein mei3
Ribosome biogenesis			
Imp4	0.553	Down	U3 snornp-associated protein imp4 (predicted)
Bfr2	0.5971	Down	Traub family protein involved in ribosome biogenesis (predicted)
S.pombe Specific protein/Conserved Fungal Protein/other proteins			
SPAC13G6.13	3.6752	Up	*Schizosaccharomyces pombe* specific protein
SPAC17A2.10c	2.4514	Up	*Schizosaccharomyces pombe* specific protein
SPAC23H3.15c	1.6264	Up	Schizosaccharomyces specific protein
Mug114	3.299	Up	*Schizosaccharomyces pombe* specific protein mug114
SPAC750.04c	1.6244	Up	*S. Pombe* specific 5tm protein family
Mug45	0.5398	Down	Schizosaccharomyces specific protein mug45
SPAC27D7.09c	1.5885	Up	But2 family protein
SPAC4F10.17	2.2899	Up	Conserved fungal protein
Mug14	1.8912	Up	Adducin
Wtf20	1.743	Up	Wtf element wtf20
Wtf18	1.7427	Up	Wtf element wtf18
Wtf4	1.6105	Up	Wtf element wtf4
Wtf5	1.8878	Up	Wtf element wtf5
Wtf9	1.5352	Up	Wtf element wtf9
Spac4h3.12C	4.1804	Up	Putative uncharacterized protein
Wtf2	0.4369	Down	Pseudogene
Spbc3d6.01	2.1877	Up	Unassigned
Mitochondrial			
Spmit.03	1.5343	Up	Mitochondrial dna binding endonuclease (predicted)
Cob1	3.2741	Up	Cytochrome b, cob1 (predicted)
Spmit.06	3.2834	Up	Mitochondrial dna binding endonuclease (predicted)

The list is organized according to gene function.

Overview of the microarray analysis

After treatment with NaF in concentrations of 30 μM or 300 mM for 0.5 h, total RNA was extracted for DNA hybridization. Our statistical analysis method (SAM) showed that 106 and 117 genes were differentially expressed in comparison with untreated controls (FDR < 0.1) in 30 μM and 300 mM NaF respectively. Genes listed in **Tables 1** and **2** were both differentially expressed with FDR < 0.1 and showed fold change of 1.5 or greater, following treatment with 30 μM or 300 mM NaF. Expression levels of 35 of the genes listed in **Table 1** were elevated by 1.5-fold or more, and 25 genes were repressed by more than 1.5-fold (FDR < 0.1). For the comparison of 300 mM NaF versus untreated control, expression of 68 genes changed more than 1.5-fold (FDR < 0.1; **Table 2**), of which 49 genes were induced and 19 genes were repressed.

For the genes upregulated by NaF, expression was elevated by 4.88-fold, while for the inhibited genes, the expression was decreased by 4.4-fold. As shown in **Figure 2A** and **2B**, cluster 3.0 was applied to cluster genes with expression levels that were elevated or decreased by at least 1.5-fold, and the results of the biological replicates were similar. Eight genes were

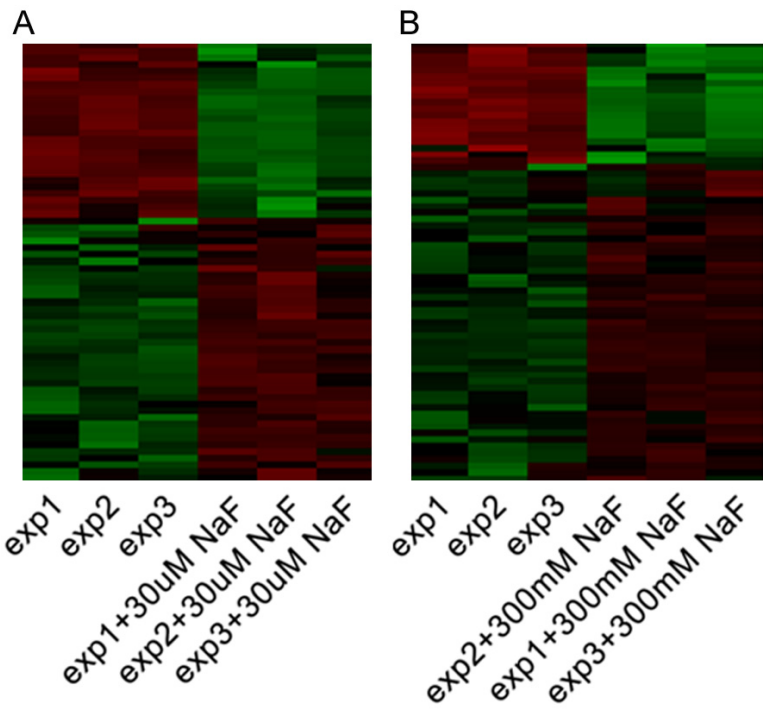

Figure 2. Hierarchical cluster of genes whose expression changed more than 1.5-fold (FDR < 0.1) in response to 30 μM (A) or 300 mM NaF (B). All treatments were performed in three replicates. The samples that were not treated with NaF are labeled as exp1, exp2, and exp3, whereas samples treated with NaF (30 μM or 300 mM) are labeled as exp1+ NaF, exp2+ NaF, and exp3 NaF.

Table 3. Confirmation of microarray data by real-time PCR

Gene symbol	Microarray		Real Time PCR	
	Fold Change	Regulation	Fold Change	Regulation
Str3	0.47	Down	0.4	Down
Ctu2	0.45	Down	0.53	Down
Nep2	3.16	Up	1.82	Up
Ght1	2.08	Up	2.9	Up
Oca2	2.17	Up	3.32	Up
Zwf2	2.85	Up	3.42	Up
Ght5	3.62	Up	3.65	Up
Spac2h10.01	4.71	Up	3.69	Up

selected randomly from those with elevated (6/8) or decreased (2/8) expression for validation with real-time PCR. Results listed in **Table 3** showed reasonable consistency in fold change between microarray and real-time PCR.

Functional classification of differentially expressed genes in response to 30 μM and 300 mM NaF

We analyzed the functional classification of differentially expressed genes whose expression level changed by greater than 1.5-fold (**Figure 3A**, **3B**) using PANTHER. Functional classifications of genes are shown in **Tables 1** and **2**. In **Table 1**, 60 genes with varying expression were classified into groups with known or unknown functional groups. The functional classifications of the 68 genes that responded to 300 mM NaF (**Table 2**) were similar to those in the 30 μM treatment experiment. Interestingly, many of the genes that responded to NaF treatment are pseudo genes or those encoding hypothetical proteins or dubious proteins.

Comparison between the 30 μM NaF response and the 300 mM NaF response

ing significantly different expression in both treatments showed opposite trends of regulation in 30 μM and 300 mM NaF treatment (**Table 4** and **Figure 5A**). These were all classified into the of category of 'Other Catalytic Activity'. TTo further gain insight into the response of *S. pombe* cells after 30 μM and 300 mM NaF treatment, we carried out a detailed comparison. As shown in **Figure 4**, expression of 41 genes changed in both 30 μM and 300 mM NaF response, whereas expression of 19 genes varied only in the presents of 30 μM NaF, and treatment with 300 mM NaF resulted in changes in expression of 26 genes specifically. The genes falling in these three categories are listed in **Tables 4**-**6** respectively.

The extent of fold change in expression wasn't significantly different among the 41 overlapping genes between the 30 μM and 300 mM NaF treatments ($p > 0.05$; **Table 4**). Five of the 41 genes showhis is an interesting observation - the mechanism of how cell sense and respond to varying NaF concentration deserves further investigation.

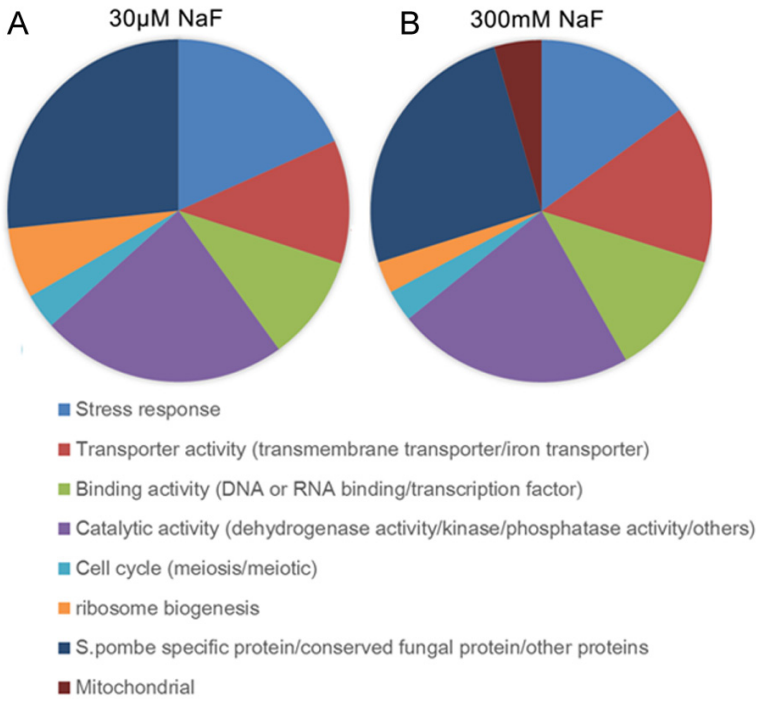

Figure 3. Pie chart showing the percentage of different functional groups in the category of molecular function. Functional classification of differentially expressed genes in response to 30 μM and 300 mM NaF.

Tables 5 and **6** show gene expression fold changes specific to 30 μM and 300 mM NaF treatments. For the 30 μM NaF treatment, three genes (SPAC212.03, SPAP27G11.11c, and SPBCPT2R1.06c) in the category of 'other protein' showed differing fold change between the two NaF dosages with same trend of regulation. Of the genes differentially expressed following 300 mM NaF treatment, all showed the same trend of regulation as seen in the 30 μM experiment, with the exception of two mitochondrial genes (SPMIT.03 and SPMIT.05) (**Table 6** and **Figure 5B**).

Comparison of the responses to NaF and environmental stress

In order to explore the commonalities between the responses to NaF and environmental stress in general, we carried out comparisons between core environmental stress response (CESR) genes with expression elevated by 1.5-fold or more, and those elevated by 2-fold or more (**Figure 6A** and **6B**).

For the 30 μM NaF treatment, the numbers of overlapping genes between these groups are shown in the Venn diagrams (**Figure 6A**). The quantity of overlapping genes in the 30 μM NaF treatment (**Table 7**) was used to evaluate the statistical significance of overlap between groups (Fisher Test). There is some overlap between genes induced by 30 μM NaF and CESR genes (p=0.000123), and genes that are induced by hydrogen peroxide (p=0), cadmium ($p=2E^{-06}$), heat shock ($p=1E^{-06}$), sorbitol (p=0.008675), and MMS (p=0.007197). Results indicated no statistical significance in differences of overlap between genes repressed by NaF (30 μM) and MMS, but 30 μM NaF induced genes were also induced by hydrogen peroxide, 25.6% by cadmium, 25.6% by heat, 14.2% by sorbitol, and 17.1% by MMS.

For the 300 mM NaF treatment, the Venn diagrams in **Figure 6B** show the numbers of overlapping genes between NaF treatment and the five environmental stresses considered above. The list of overlapping genes is presented in **Table 7**; statistical significance was assessed by Fisher's Test ($P < 0.05$). We observed considerable overlap between genes induced by 300 mM NaF and CESR genes (p=0.049812), genes that are induced in hydrogen peroxide (p=0), cadmium ($p=3.4E^{-05}$), heat shock (p=0), sorbitol (p=0.005287) and MMS (p=0.011284).

Discussion

In this study, we analyzed the global gene expression profile response to NaF in *S. pombe* cells. The functional classification of differentially expressed genes in response to 30 μM or 300 mM of NaF are reported. We found that NaF causes differential gene expression in transporters, stress response proteins and transcription factors. Differences and similarities of functional classification in genes responding to 30 μM and 300 mM NaF were analyzed. Comparison between the NaF response and environmental stress response showed NaF-specificity for many of the genes we identified. We therefore anticipate that this study should enrich understanding of the molecular functions of NaF in cells.

Table 4. Genes showing overlap between the 30 μM and 300 mM NaF treatment groups

Gene symbol	Regulation (30 μM)	Fold change (30 μM)	Regulation (300 mM)	Fold change (300 mM)	Gene products
Stress response					
SPAC19D5.01	Up	1.8216	Up	1.8106	Tyrosine phosphatase Pyp2
SPACUNK4.17	Up	1.7131	Up	1.6513	NAD binding dehydrogenase family protein
SPBC660.05	Up	3.0347	Up	2.2035	WW domain containing conserved fungal protein
SPBC725.03	Up	1.5227	Up	1.515	Pyridoxamine 5'-phosphate oxidase (predicted)
SPCC1020.10	Up	2.0162	Up	2.172	Serine/threonine protein kinase Oca2
SPCC1223.03c	Up	1.9038	Up	1.6952	Glycerol-3-phosphate dehydrogenase Gut2 (predicted)
SPCC1739.05	Down	0.5381	Down	0.6125	Histone lysine methyltransferase Set5 (predicted)
Transporter Activity (transmembrane transporter/iron transporter)					
SPAC1751.01c	Up	1.6527	Up	1.8557	Gluconate transmembrane transporter inducer Gti1
SPAC1F8.03c	Down	0.47	Down	0.4742	Siderophore-iron transporter Str3
SPAC24H6.13	Down	0.6611	Down	0.5957	DUF221 family protein implicated in Golgi to Plasma membrane transport (predicted)
SPCC1235.14	Up	3.5221	Up	3.6205	Hexose transmembrane transporter Ght5
SPCC548.07c	Up	2.2091	Up	2.0812	Hexose transmembrane transporter Ght1
SPAC1F8.06	Down	0.5928	Down	0.647	Cell surface glycoprotein, flocculin Pfl8
Binding activity (DNA or RNA binding/transcription factor)					
SPAC16.05c	Down	0.5406	Down	0.546	Transcription factor Sfp1 (predicted)
SPAC2H10.01	Up	4.4577	Up	4.7135	Transcription factor, zf-fungal binuclear cluster type (predicted)
SPCC1223.13	Up	1.6599	Up	1.6674	CBF1/Su(H)/LAG-1 family transcription factor Cbf12
SPAC683.02c	Down	0.5505	Down	0.5714	Zf-CCHC type zinc finger protein (predicted)
SPAPB1A10.14	Up	2.5744	Up	2.711	F-box protein (predicted)
SPBC16E9.16c	Up	2.2633	Up	1.7524	Lsd90 protein
Catalytic Activity (dehydrogenase activity/kinase/phosphatase activity/others)					
SPAC25B8.13c	Down	0.6444	Down	0.6107	2-OG-Fe(II) oxygenase superfamily protein
SPBC106.02c	Down	0.6121	Down	0.6383	Sulfiredoxin
SPCC1450.10c	Down	0.5913	Down	0.5974	Iron hydrogenase Nar1 (predicted)
SPCC1739.08c	Up	1.8985	Up	1.8354	Short chain dehydrogenase (predicted)
SPCC794.01c	Up	2.5825	Up	2.8519	Glucose-6-phosphate 1-dehydrogenase Zwf2 (predicted)
SPAC1F5.09c	Up	1.757	Up	1.792	PAK-related kinase Shk2
SPBC19C2.13c	Up	2.1257	Down	0.4546	Cytosolic thiouridylase subunit Ctu2
SPCC1020.01c	Up	2.7506	Down	0.6186	P-type proton ATPase, P3-type Pma2
SPBC32H8.02c	Down	0.461	Up	3.1579	NEDD8 protease Nep2
SPBC336.02	Up	1.5675	Down	0.4869	18S rRNA dimethylase (predicted)
SPBPB7E8.02	Down	0.4703	Up	1.6131	PSP1 family protein
Cell Cycle (meiosis/meiotic)					
SPAC15E1.07c	Up	2.6792	Up	2.7484	Meiotic kinetochore protein (Meikin) Moa1
Ribosome biogenesis					
SPAC19A8.07c	Down	0.6237	Down	0.553	U3 snoRNP-associated protein Imp4 (predicted)
SPAC664.08c	Down	0.545	Down	0.5971	Traub family protein involved in ribosome biogenesis (predicted)
S.pombe Specific protein/Conserved Fungal Protein/other proteins					
SPAC17A2.10c	Up	4.3801	Up	2.4514	*Schizosaccharomyces pombe* specific protein
SPAC23H3.15c	Up	2.567	Up	1.6264	*Schizosaccharomyces* specific protein
SPAC4F8.08	Up	2.5958	Up	3.299	*Schizosaccharomyces pombe* specific protein Mug114
SPBP8B7.04	Down	0.6429	Down	0.5398	*Schizosaccharomyces* specific protein Mug45
SPAC4F10.17	Up	2.1388	Up	2.2899	Conserved fungal protein
SPBC359.06	Up	1.685	Up	1.8912	Adducin
SPCC1906.04	Up	1.927	Up	1.743	Wtf element Wtf20
SPCC548.03c	Up	1.8948	Up	1.6105	Wtf element Wtf4

In our study, genes encoding different proteins with varying functions were classified as follows: (1) Eleven genes are identified with known or predicted function in stress responses; (2)

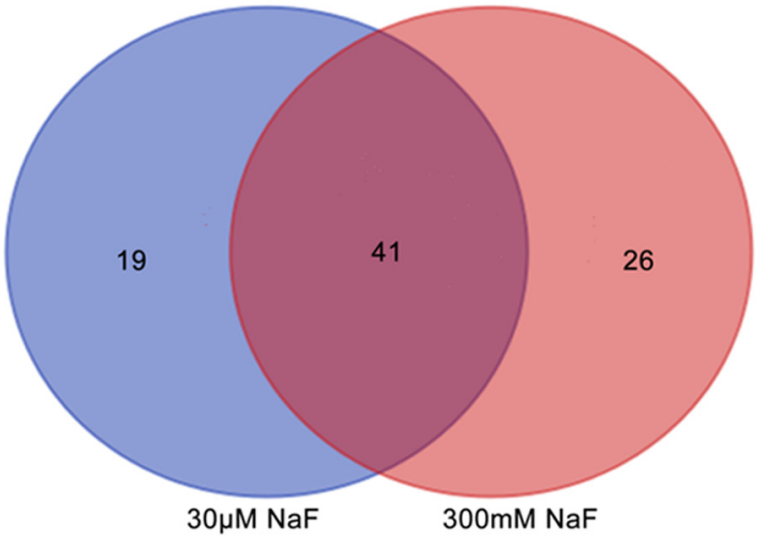

Figure 4. Venn diagram representing the overlap of gene sets under 30 μM and 300 mM NaF treatment. Numbers refer to gene that belong to each group. Forty-one genes were differentially expressed following treatment with both concentrations of NaF.

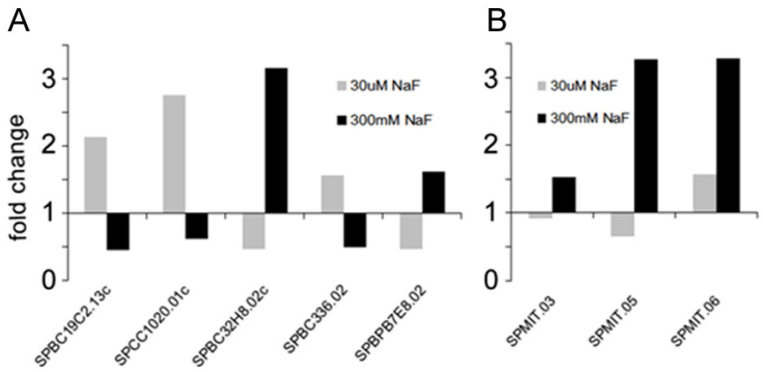

Figure 5. Bar graphs showing opposing trends in fold change in gene expression for 30 μM and 300 mM NaF treatments.

Seven genes encode putative or known transporters including siderophore iron transporter Str3, plasma membrane transporter (SPAC 24H6.13, which is a DUF221 family protein involved in Golgi to plasma membrane transport), and four transmembrane transporters which function as gateways to permit gluconate, urea and hexose across the biological membrane (Gti1, SPAC869.03c, Ght1 and Ght5). Fluoride may relate to pathologies through its effect on secretion and vesicular traffic via transport proteins that are synthesized in the ER and subsequently transported to the Golgi and plasma membrane [9]. All the transporters identified in our Gene Ontology Term analysis are in the category of integral components of the plasma membrane. Interestingly, fluoride sensing riboswitch genes coding for fluoride channels Fex 1 and Fex 2 did not respond to NaF, but the expression level of FEX was consistently high in 0, 30 μM and 300 mM NaF conditions.

The mechanism and function of NaF in *S. pombe* cells require further investigation. (3) Of the genes we identified being NaF-responsive, three encode transcription factors. (4) Three other genes encode DNA or RNA binding proteins. (5) Fourteen encode genes with catalytic activities including dehydrogenase, kinase, phosphatase, and dimethylase. (6) Two meiosis or meiotic cell cycle related genes were also identified. (7) Four genes encoding proteins involved in ribosome biogenesis were all downregulated. (8) The other genes we identified encodefive*S.pombe*-specificproteins, five conserved fungal proteins, and six other uncharacterized proteins.

It has been reported that membrane transporters are associated with transportation of NaF [17, 18]. Although in our experimental system we used 30 μM or 300 mM NaF to treat cells, we did not measure the real intercellular concentration of NaF. Our observation that fold changes of the 41 genes identified in both 30 μM and 300 mM NaF conditions are similar indicate that the intracellular response to NaF may be largely indifferent to concentration. However, it is notable that the 30 min treatment time we used is short in comparison to fission yeast cell generation time, and therefore likely to be too short to cause substantially detrimental effects on gene expression.

Genes with varying expression levels in response to five different environmental stresses

Table 5. Genes that showed altered expression specifically in response to 30 μM NaF treatment

Gene symbol	Regulation (30 μM)	Fold Change (30 μM)	Regulation (300 mM)	Fold Change (300 mM)	Gene Products
Stress response					
Psu1	Down	0.5727	Down	0.6801	Cell wall protein Psu1, beta-glucosidase (predicted)
Spac19b12.01	Down	0.6234	Down	0.7095	TPR repeat protein, TTC27 family
Rrp9	Down	0.659	Down	0.6921	U3 snoRNP-associated protein Rrp9 (predicted)
Cox3	Up	1.6161	Up	1.2636	Cytochrome c oxidase 3 (predicted)
Transporter activity (transmembrane transporter/iron transporter)					
SPAC869.03c	Up	1.5235	Up	1.6479	Urea transmembrane transporter (predicted)
Catalytic activity (dehydrogenase activity/kinase/phosphatase activity/others)					
SPAC22A12.17c	Up	1.7317	Up	1.4453	Short chain dehydrogenase (predicted)
Ark1	Down	0.627	Down	0.7185	Aurora-B kinase Ark1
Alp41	Up	1.545	Up	1.248	GTP-binding protein involved in beta-tubulin folding Alp41
Cell cycle (meiosis/meiotic)					
Dic1	Down	0.6284	Down	0.7048	Meiotic dynein intermediate chain Dic1
Ribosome biogenesis					
Sda1	Down	0.6021	Down	0.6973	SDA1 family protein (predicted)
Rrp1402	Down	0.6638	Down	0.7445	Ribosome biogenesis protein Rrp14 (predicted)
S.pombe specific protein/Conserved Fungal Protein/other proteins					
SPAPJ695.01c	Down	0.5917	Down	0.8465	*S. pombe* specific UPF0321 family protein 3
SPAC1952.04c	Up	1.6079	Up	1.3879	Conserved fungal protein
SPAC212.03	Up	3.0055	Up	1.7681	Hypothetical protein
SPAP27G11.11c	Down	0.3035	Down	0.7917	Dubious
SPBCPT2R1.06c	Down	0.2018	Down	0.8626	Unassigned
SPBPB21E7.08	Up	1.625	Up	1.5739	Unassigned
Wtf3	Up	3.1686	Up	2.2707	Pseudogene
Wtf24	Up	1.6178	Up	1.4358	Pseudogene

Table 6. Genes that showed altered expression specifically in response to 300 mM NaF treatment

Gene symbol	Regulation (300 mM)	Fold Change (300 mM)	Regulation (30 mM)	Fold Change (30 mM)	Gene Products
Stress Responsing					
SPAPB1A11.03	Up	1.5249	Up	1.1729	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase) (predicted)
SPBC1289.14	Up	1.5515	Up	1.3096	Adducin (predicted)
SPCC191.01	Up	1.5286	Up	1.4839	*Schizosaccharomyces* specific protein
Transporter activity (transmembrane transporter/iron transporter)					
vta1	Up	1.8167	Up	1.0823	Vps20 associated protein Vta1 (predicted)
Isp4	Down	0.642	Down	0.6725	OPT oligopeptide transmembrane transporter family Isp4
SPCC794.04c	Up	1.5674	Up	1.2063	Amino acid transmembrane transporter (predicted)
Binding activity (DNA or RNA binding/transcription factor)					
Hsp3105	Up	1.8328	Up	1.3638	ThiJ domain protein
SPBC16C6.03c	Up	1.5171	Up	1.298	Ribosome assembly protein (predicted)
Catalytic Activity (dehydrogenase activity/kinase/phosphatase activity/others)					
SPAC186.07c	Down	0.3884	Down	0.3962	Hydroxyacid dehydrogenase (predicted)
Pik1	Up	1.8212	Down	0.9975	1-phosphatidylinositol 4-kinase Pik1
Fip1	Down	0.6605	Down	0.6768	Iron permease Fip1
SPAC869.04	Up	5.6954	Up	2.8507	Formamidase-like protein (predicted)
SPBC2G2.17c	Up	1.6601	Up	1.4058	Beta-glucosidase Psu2 (predicted)
Cell Cycle (meiosis/meiotic)					
SPBC119.04	Up	3.3802	Up	2.6655	Meiosis inducing protein Mei3
S. pombe Specific protein/Conserved Fungal Protein/other proteins					
SPAC13G6.13	Up	3.6752	Up	1.1817	*Schizosaccharomyces pombe* specific protein
SPAC750.04c	Up	1.6244	Up	1.4872	*S. pombe* specific 5Tm protein family
SPAC27D7.09c	Up	1.5885	Up	1.3953	But2 family protein
SPCC285.07c	Up	1.7427	Up	1.4286	Wtf element Wtf18

SPCC794.02	Up	1.8878	Up	1.5346	Wtf element Wtf5
SPCC970.11c	Up	1.5352	Up	1.4903	Wtf element Wtf9
SPAC4H3.12c	Up	4.1804	Up	2.8087	Putative uncharacterized protein
SPBC1706.02c	Down	0.4369	Down	0.9833	Pseudogene
SPBC3D6.01	Up	2.1877	Up	1.6914	Unassigned
Mitochondrial					
SPMIT.03	Up	1.5343	Down	0.9189	Mitochondrial DNA binding endonuclease (predicted)
SPMIT.05	Up	3.2741	Down	0.6543	Cytochrome b, Cob1 (predicted)
SPMIT.06	Up	3.2834	Up	1.5739	Mitochondrial DNA binding endonuclease (predicted)

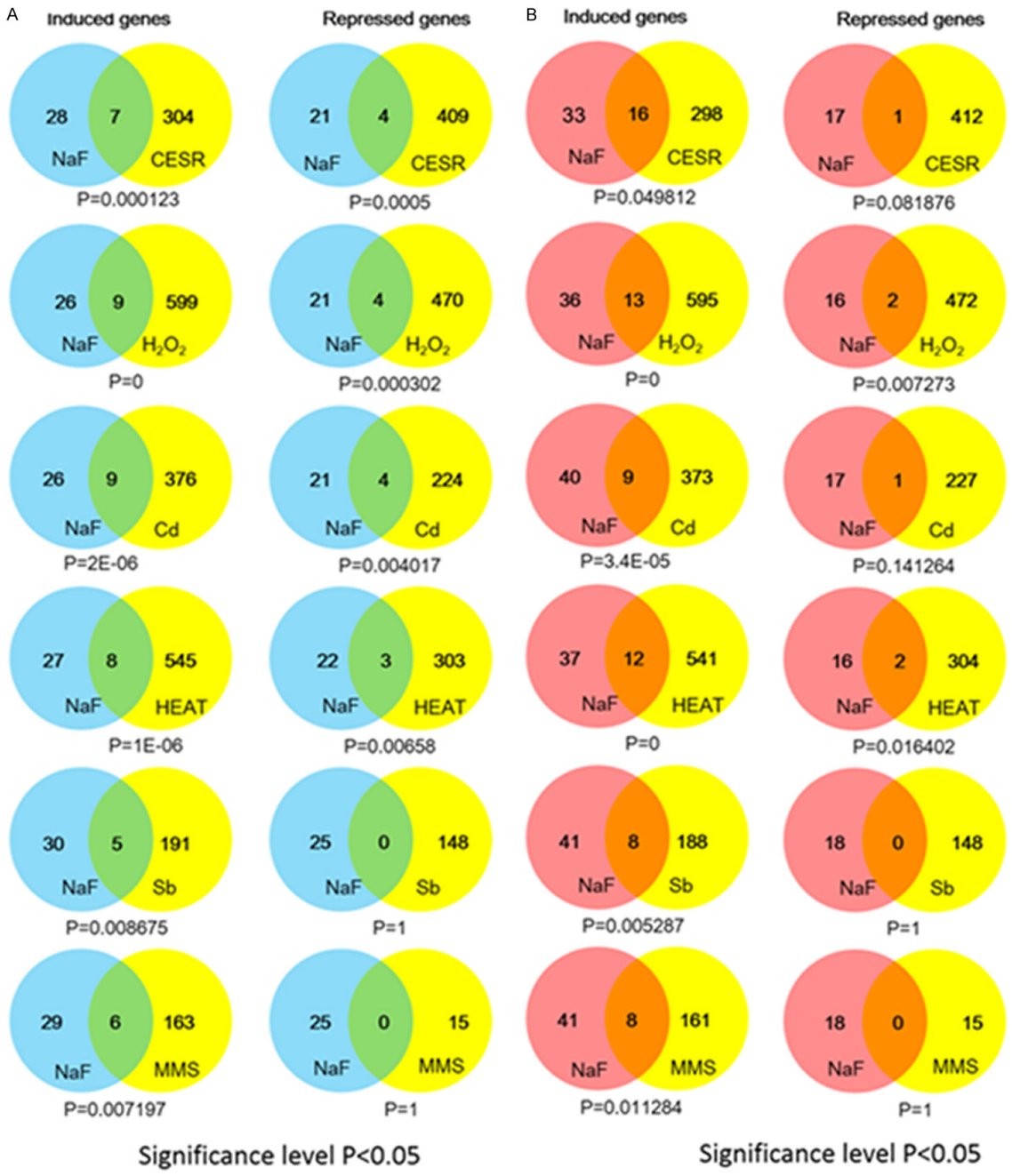

Figure 6. Comparison between the NaF response and the environmental stress response. Comparison between the genes induced or repressed more than 1.5-fold (FDR, < 0.1; A) and genes differentially expressed by more than 2-fold (FDR, < 0.1; B) in response to NaF and CESR genes are illustrated by Venn diagrams. Statistical significance was inferred where $p < 0.05$.

Table 7. Overlapping genes that showed altered expression in response to NaF treatment and 5 environmental stresses

	30 μM NaF		300 mM NaF	
	Induced genes	Repressed genes	Induced genes	Repressed genes
CESR	Spacunk4.17	SPAC19B12.01	SPACUNK4.17	set5
	Spbc660.05	Psu1	SPAPB1A11.03	
	Spbc725.03	Rrp9	SPBC1289.14	
	Cox3	Set5	SPBC660.05	
	Gut2		SPBC725.03	
	Oca2		SPCC191.01	
	Pyp2		Gut2	
			Oca2	
			Pyp2	
H_2O_2	Spac2h10.01	SPAC19B12.01	SPAC2H10.01	set5
	Spac4f10.17	SPBC336.02	SPAC4F10.17	SPBC336.02
	Spacunk4.17	Rrp9	SPACUNK4.17	
	Spbc660.05	Set5	SPAPB1A11.03	
	Spbc725.03		SPBC1289.14	
	Cbf12		SPBC660.05	
	Mug14		SPBC725.03	
	Oca2		SPCC191.01	
	Pyp2		Cbf12	
			Mug14	
			Oca2	
			Pyp2	
			Wtf5	
HEAT	Spac4f10.17	Set5	SPACUNK4.17	set5
	Spacunk4.17	SPBC336.02	SPAC4F10.17	SPBC336.02
	Spbc660.05	SPAC19B12.01	SPCC191.01	
	Spbc725.03		SPBC1289.14	
	Spbpb7e8.02		SPBC725.03	
	Mug14		SPBC660.05	
	Oca2		SPBC359.06	
	Pyp2		SPAPB1A11.03	
			SPBPB7E8.02	
			SPAC19D5.01	
			SPBC119.04	
			SPCC1020.10	
Cd	Spacunk4.17	SPAC19B12.01	SPACUNK4.17	SPBC336.02
	Spac19d5.01	SPAC2E1P5.05	SPAC19D5.01	
	Spbc725.03	SPBC947.07	SPCC191.01	
	Spbc660.05	SPBC336.02	SPBC1289.14	
	Spbpb7e8.02		SPBC725.03	
	Spcc1020.10		SPBC660.05	
			SPAPB1A11.03	
			SPBPB7E8.02	
			SPCC1020.10	
Sb	Spacunk4.17	/	SPACUNK4.17	/
	Spbc660.05		SPBC660.05	

(hydrogen peroxide, cadmium, heat, sorbitol and MMS) are defined as the core environmental stress response (CESR) genes [19]. Our results revealed that NaF induces expression changes in these CESR genes, which is consistent with the notion that NaF at non-physiological concentrations can be regarded as a type of environmental stress for cells. We note that 300 mM NaF induces more genes than 30 μM NaF. NaF as a type of salt is predicted to cause osmotic stress to cells at high concentration and, therefore, causes a similar response to sorbitol as an environmental stress. According to our data, there are 8 genes induced by 300 mM NaF that are also induced by sorbitol stress out of 49 induced genes, suggesting that the toxicity of NaF at 300 mM may have a more dominant effect. In addition, stress response genes are secondary to genes related to growth rates [20]. This phenomenon has also been observed in the Atf1/Pcr1 transcriptional response to oxidative stress in fission yeast [21]. Only three genes encoding transcription factors were found among the 15 H_2O_2 stress responsive genes identified here, indicating the toxicity of NaF was stronger than that of other stressors.

In conclusion, our results showed that NaF causes differential expression of genes encoding transporters, stress response proteins, and transcription factors. Comparison of the responses to NaF and environmental stress revealed that many stress response genes were specific to NaF.

	SPAC19D5.01		SPAC19D5.01	
	SPBC725.03		SPAPB1A11.03	
	SPCC1020.10		SPCC191.01	
			SPBC1289.14	
			SPBC725.03	
			SPCC1020.10	
MMS	SPACUNK4.17	/	SPACUNK4.17	/
	SPBC660.05		SPBC660.05	
	SPAC19D5.01		SPAC19D5.01	
	SPAPB1A10.14		SPAPB1A10.14	
	SPBC725.03		SPCC191.01	
	SPCC1020.10		SPBC1289.14	
			SPBC725.03	
			SPCC1020.10	

These results suggest that NaF induces global gene expression changes in *S. pombe* cells, and this finding is expected to improve the general understanding of the molecular function of NaF in cells.

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Disclosure of conflict of interest

None.

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Table S1. Sequences of primers used in real-time PCR

Gene ID	Forward (5'-3')	Reverse (5'-3')
SPCC794.01c	ACCTTGTCCCATGTTTGCGG	ACCTTGTCCCATGTTTGCGG
SPBC19C2.13c	GCGGTCTGCGATTCATGCTT	ACCACCGGAAATAGCCAGCA
SPCC1235.14	GATGGATGTTTGGCGCCGAT	CCGCGCCGAAGAATACGAAT
SPCC1020.01c	ACTGCCGCACCTAACACTCA	AGGACGGGCAGGCTTTTCAT
SPAC1F8.03c	CGGAGGAGAAAAGCGAAAATGGA	TGGTATTGTAGCCCGATCCT
SPCC1020.10	GAACCTGTTTCTCGTCGTCT	TGTTGGACTATGACTGACGGGG
SPBC23E6.06c	CGATGAAACGCGTGAAAACG	CAGTCATGGGTACGCAGACA
SPAC25B8.04c	CCATCCTTTATCGCGTTCTCCC	TTCCCGAAATTGGACACCGA
SPCC548.07c	GCAGTATCCCCGCATTTGGT	CCTGCGAAGGGTAAGTTTAGGA
SPAC2H10.01	TGTGCTCACTTTCTCGCTCA	AAGGGAAGGATTGACTCGGC
SPBC17D11.04c	CAGCCGAGAGAAGAGATTCCCT	AAGAATCACGAAGGCCTGCA
SPBC32H8.02c	CCACCCATCTCCATCTTCACCT	AAAAGGAGCCGGCAATGACC
SPMIT.01	TGCTCCAGATGTTGCTTACCCT	ATACCGTCCAACCACCACCA
β-actin	CCGGTATTCATGAGGCTACT	GGAGGAGCAACAATCTTGAC