Original Paper

The JNK-Like MAPK KGB-1 of *Caenorhabditis Elegans* Promotes Reproduction, Lifespan, and Gene Expressions for Protein Biosynthesis and Germline Homeostasis but Interferes with Hyperosmotic Stress Tolerance

Peter Gerke Alex Keshet Ansgar Mertenskötter Rüdiger J. Paul

Institute of Zoophysiology, University of Münster (WWU), Münster, Germany

Key Words

Activator protein 1 • Chaperone • FOS • Insulin/IGF receptor ortholog • JUN • Regulatory volume increase • Sperm • Target of rapamycin (TOR)

Abstract

Aims: This study focused on the role of the JNK-like MAPK (mitogen-activated protein kinase) KGB-1 (kinase, GLH-binding 1) for osmoprotection and other vital functions. **Methods:** We mapped KGB-1 expression patterns and determined lifespan, reproduction and survival rates as well as changes in body volume, motility, and GPDH (glycerol-3-phosphate dehydrogenase) activity for glycerol production in wildtype (WT), different signaling mutants (including a *kbg-1* deletion mutant, *kbg-1Δ*) and RNAi-treated worms under control and hyperosmotic conditions. KGB-1-mediated gene expressions were studied, for instance, by RNA Sequencing, with the resulting transcriptome data analyzed using orthology-based approaches. **Results:** Surprisingly, mutation/RNAi of *kbg-1* and *fos-1* (gene for an AP-1, activator protein 1, element) significantly promoted hyperosmotic resistance, even though hyperosmotic GPDH activity was higher in WT than in *kbg-1Δ*. KGB-1 and moderate hyperosmolarity promoted and severe hyperosmolarity repressed *kbg-1*, *fos-1*, and *jun-1* (gene for another AP-1 element) expression. Transcriptome profiling revealed, for instance, down-regulated genes for protein biosynthesis and up-regulated genes for membrane transporters in *kbg-1Δ* and up-regulated genes for GPDH-1 or detoxification in WT, with the latter indicating cellular damage and less effective osmoprotection in WT. **Conclusion:** KGB-1 promotes reproduction and lifespan and fosters gene expressions for AP-1 elements, protein biosynthesis, and balanced gametogenesis, but inhibits expressions for membrane transporters perhaps in order to control energy consumption. Reduced protein biosyntheses and enhanced membrane transports in *kbg-1Δ* most likely contribute to the high hyperosmotic tolerance of the mutant by easing the burden of the existing chaperone machinery and promoting regulatory volume increases upon hyperosmotic stress.

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Rüdiger J. Paul

Institute of Zoophysiology, University of Münster (WWU),
Schlossplatz 8, D-48143 Münster (Germany)
Tel. +49 251 83 23851, Fax +49 251 83 23876, E-Mail paulr@uni-muenster.de

Introduction

Like in other animals, *C. elegans* cells (and the worm itself) lose water and shrink upon hyperosmotic exposure but regain their initial volume within a few tens of minutes [1]. Nevertheless, rapid and extensive protein damage may occur depending on the intensity of the hyperosmotic stress [2]. Later cell homeostasis is achieved in this species by de novo synthesis of glycerol, with GPDH catalyzing its rate-limiting step and accumulated misfolded proteins triggering *gpdh-1* expression [3]. Insulin/IGF-like signaling and DAF-16-mediated gene expressions for trehalose synthesis enzymes have also been reported to contribute to osmoprotection in *C. elegans* [4] such as imports of another compatible osmolyte by the H⁺-dependent myo-inositol transporter HMIT-1.1 [5]. To minimize protein damage, protein biosynthesis rate may be lowered to reduce the risk of overloading the existing chaperone machinery [6]. As the regulatory pathways for cellular hyperosmotic stress responses are not yet understood in detail [1], we focused on MAPK signaling pathways, which can be considered as ancient transducers of osmosensory signals [7]. Evolution has caused intense variation in their structure and function resulting in MAPK processing of many kinds of signals and MAPK control of various physiological processes. Among the three subgroups of MAPKs, p38 kinases and c-Jun N-terminal kinases (JNK) are frequently involved in stress responses of animal cells [8]. *C. elegans* expresses JNK and JNK-like MAPKs in a tissue-specific way, with JNK-1 and its upstream kinase, the MAP2K JKK-1, expressed only in neuronal cells [9, 10]. The signaling pathway of the JNK-like MAPK KGB-1 is composed of MLK-1 (MAP3K), MEK-1 (MAP2K), and KGB-1 [11, 12]. This cascade is controlled by MAX-2 (a Ste20-related kinase activating MLK-1) and MIG-2 (a Rac-type small GTPase activating MAX-2) [13]. The MAPK phosphatase VHP-1 negatively regulates the KGB-1 pathway by dephosphorylating KGB-1 [11, 14], and the scaffold protein SHC-1 binds MLK-1 and MEK-1 to promote the phosphorylation of MEK-1 and later KGB-1 [12]. Perhaps, the SEK-1/PMK-1 p38-type MAPK pathway [15] also affects KGB-1 activity [16]. The expression of *mek-1* in neurons and of *mlk-1*, *mek-1*, and *shc-1*, for instance, in muscles, hypodermis, intestine, pharynx, and uterine endothelial cells [12, 17, 18] as well as KGB-1 expression in germ cells (oocytes) [16] indicate important roles of KGB-1 signaling for many different functions. KGB-1 has been reported to interact with germline RNA helicases (GLHs 1–4) [16, 19], which are constitutive components of germ cell-specific P granules (aggregates of RNA and proteins) that are required for germ line proliferation, oogenesis, and fertility [20–22]. It was also suggested that KGB-1 and CSN-5 (a subunit of the COP9 signalosome, which regulates protein stability [23]) control germline homeostasis. Mutations of *mlk-1*, *mek-1*, and *kgb-1*, on the other hand, caused a reduced tolerance to heavy metals [11, 12, 17]. Another study [18] showed a reduced tolerance of *shc-1Δ* to heavy metals, heat, and oxidative stress as well as effects of SHC-1 on DAF-2 (dauer formation 2) signaling [24] resulting in a reduced nuclear localization of DAF-16 in *shc-1Δ*. KGB-1 has recently been shown to phosphorylate FOS-1 (a bZIP transcription factor) to derepress heavy metal tolerance genes, which are repressed by unphosphorylated FOS-1 [25]. Further on, positive effects of the *Bacillus thuringiensis* toxin Cry5B on *kgb-1* transcription have been reported, just as a reduced tolerance of *kgb-1Δ* (and *mek-1Δ*) to Cry5B [26] or *Pseudomonas aeruginosa* [14]. The underlying KGB-1-mediated defense pathway against pore-forming toxins from soil bacteria involves again bZIP transcription factors (JUN-1, FOS-1), which contribute to the forming of AP-1 complexes [27]. KGB-1/AP-1 signaling (i.e., KGB-1-mediated phosphorylation of JUN-1 and FOS-1) also constitutes a fasting-responsive pathway, which promotes longevity through regulating proteostasis (i.e., up-regulation of the SCF E3 ligase complex and activation of the ubiquitin proteasome system to degrade damaged or unused proteins) and collaborating with nuclear DAF-16 [28]. Linked to an age-dependent antagonistic modulation of DAF-16, KGB-1 function changes with age [29], causing stress protection in developing larvae but a lower resistance to heavy metals and protein folding stress in young adults.

After detecting effects of KGB-1 on hyperosmotic tolerance in *C. elegans*, we studied KGB-1 expression patterns, checked for contributions of KGB-1 and insulin/IGF-like (DAF-2)

signaling to hyperosmotic resistance and its mechanisms, and analyzed effects of KGB-1 and hyperosmolarity on gene expression. Expression patterns were studied using fluorescent protein reporter genes. Indications for an elevated hyperosmotic tolerance of a *kgb-1* deletion mutant from studies on reproduction rate and lifespan initiated more detailed investigations of the effects of hyperosmolarity on survival rate, body volume and motility, and the activity of the osmolyte-producing enzyme GPDH as well as on the contributions of DAF-2 signaling to hyperosmotic tolerance. Effects of KGB-1 and hyperosmolarity on gene expressions were studied using RT-PCR, a reporter gene assay, and RNA Sequencing (RNA-Seq; next-generation sequencing). This study provides novel insights into inhibitory and activating pathways for cellular hyperosmotic stress responses, the mechanisms of osmoprotection, and the physiological role of the JNK-like MAPK KGB-1.

Materials and methods

Wildtype and mutant strains

C. elegans N2 (Bristol variety) and mutants, hereinafter referred to as *kgb-1*Δ [KB3 *kgb-1*(*um3*) IV], *jnk-1*Δ [VC8 *jnk-1*(*gk7*) IV], *pmk-1*Δ [KU25 *pmk-1*(*km25*) IV], *mek-1*Δ [FK171 *mek-1*(*ks54*) X], *sek-1*Δ [KU4 *sek-1*(*km4*) X], *daf-2*Δ [CB1370 *daf-2*(*e1370*) III], and *fos-1*Δ [GS384 *fos-1*(*ar105*)/*dpy-11*(*e224*) *unc-42*(*e270*) V], were obtained from the *Caenorhabditis* Genetics Center (CGC; <http://www.cbs.umn.edu/CGC/>). Worms were kept at 20°C on NGM with *E. coli* OP50 as food source. According to German law, experiments carried out on the invertebrate *C. elegans* do not have to be announced or approved.

Transgenic strains

The $P_{k_{gb-1}}::gfp$ plasmid was constructed by fusion of a nuclear localization signal (NLS) and the GFP coding sequence to a 1.770-kbp genomic fragment upstream of the *kgb-1* translational start codon ($P_{k_{gb-1}}$). $P_{k_{gb-1}}$ was amplified from N2 genomic DNA by PCR (*Taq*-polymerase; Segenetic, Borken, Germany) (primers: 5'-GGTGGGGTGACGATGTGTCG-3', 5'-TTCTGACCCGGGGCGTTTACCTC-3'), and the associated *Bam*HI/*Hind*III fragment was cloned into pPD95.67 (Andrew Fire, Stanford, USA). The *kgb-1::gfp* plasmid was made by fusion of the GFP coding sequence to a 1.770-kbp genomic fragment upstream of the *kgb-1* translational start codon ($P_{k_{gb-1}}$) and the KGB-1 coding sequence without the last two N-terminal codons. The cDNA of *kgb-1* was amplified by PCR (primers: 5'-AGTGGATCCGCCGTACATAACGA-3', 5'-TTGGTACCCAGTGAAAATGTCGTGGTCCG-3') and cloned into pJET1/blunt (GeneJET™ PCR Cloning Kit, Fermentas, St. Leon-Rot, Germany). *Bam*HI/*Kpn*I digestion enabled cloning of the *kgb-1* fragment (1.157 kbp) into the $P_{k_{gb-1}}::gfp$ plasmid (between $P_{k_{gb-1}}$ and *gfp* without NLS signal). The $P_{fos-1}::ecfp$ plasmid was constructed by fusion of the ECFP (enhanced cyan fluorescent protein) coding sequence to a 5.942-kbp genomic fragment upstream of the *fos-1b* translational start codon to cover at best both *fos-1b* and *fos-1a* promoters [30]. P_{fos-1} was amplified from N2 genomic DNA by PCR (Phusion® DNA polymerase; Finnzymes, Vantaa, Finland), inserting with it a 5'-*Kpn*I restriction site (primers: 5'-GGTACCATTTCATGTTT-3', 5'-TCCTCGCCCTTGCTCACCATTCCACTCTTATATAGCAG-3'). *ecfp* was amplified from the pCAG-CFP plasmid (Addgene, Cambridge, MA, USA) by PCR, inserting with it a 3'-*Bam*HI restriction site (primers: 5'-CTGCTATATAAGAGAGTGGAATGGTGAGCAAGGGCGAGGA-3', 5'-GGATCCTTACTTGTACAGCTCGTCCA-3'). Both PCR products (P_{fos-1} , *ecfp*) were fused and amplified by PCR (primers: 5'-GGTACCATTTCATGTTT-3', 5'-GGATCCTTACTTGTACAGCTCGTCCA-3') and cloned into pJET1.2/blunt. All plasmids were checked by sequencing.

The reporter strains $P_{k_{gb-1}}::gfp$ (MS10), *kgb-1::gfp* (MS11), and $P_{fos-1}::ecfp$ (MS12) were generated by injecting the corresponding plasmids at a concentration of 200 ng/μL ($P_{k_{gb-1}}::gfp$), 100 ng/μL ($P_{fos-1}::ecfp$), or 50 ng/μL (*kgb-1::gfp*) into wildtype (N2 Bristol), along with the plasmid pRF4 [100 ng/μL [31], which carried the co-injection marker *rol-6*(*su1006*) [32]. The transgenic strain TJ356 carrying $P_{daf-16}::daf-16::gfp$ was obtained from the *Caenorhabditis* Genetics Center (CGC; <http://www.cbs.umn.edu/CGC/>).

RNA interference

For RNA interference (gene knock-down), double-stranded RNA was applied by feeding the *E. coli* HT115 strains provided by Source BioScience LifeSciences (Nottingham, UK): *kgb-1*-RNAi [T07A9.3], *sek-*

1-RNAi [R03G5.2], *daf-16*-RNAi [R13H8.1], *fos-1*-RNAi [F29G9.4], and ctrl-RNAi (L4440, control, empty vector). Identity of bacteria was checked by PCR. Bacteria were grown at 37°C for 18 hours in 2xYT + Amp medium [33]. NGM plates (1 mmol/L isopropyl β -D-1-thiogalactopyranoside, IPTG; 100 μ g/mL ampicillin) were seeded with 750 μ L bacterial suspension (HT115) and allowed to grow for 24 hours at 20°C to induce dsRNA expression. Synchronized L1 larvae were seeded to these plates, grown until the (young) adult stage, and then submitted to hyperosmotic test conditions on NGM plates inoculated with the respective bacterial strain.

Microscopy

An Axiovert 100 (Zeiss, Germany), equipped with a Kappa CF 8/1 FMC CCD (Kappa optronics, Gleichen, Germany), Nikon COOLPIX 990 or Canon EOS 350D camera, was used for the fluorescence microscopy of worms, which were anesthetized with 10 mM Levamisole in M9 buffer. Image processing was done using Adobe Photoshop® (Adobe Systems Incorporated, San Jose, CA, USA) or ImageJ (v. 1.44; <http://imagej.nih.gov/ij/>). To evaluate *fos-1* expression (Fig. 6F, G), pictures of worms were taken at identical camera settings. With ImageJ, the RGB channels of a picture were splitted, and the red channel was subtracted from the blue channel. After contrast enhancement (at identical settings), the mean pixel intensity of the two posterior intestinal segments was determined. The results were normalized with respect to mean pixel intensities from control animals.

Experimental protocol I: Reproduction and lifespan of worms

To determine reproduction and lifespan, single synchronized L1 larvae were transferred to control (standard NaCl concentration, 51 mmol/L) or test (standard NaCl concentration plus 200 mmol/L NaCl) plates (NGM plates, seeded with *E. coli* OP50), and transferred every 24 hours to fresh plates to avoid mixing with offspring. Lifespan was counted from the date of bleaching worms [34]. The number of offspring per day as well as lifespan were monitored for each worm. Survival was tested by applying gentle touch stimuli. Worms leaving NGM and dying at the wall were excluded from evaluation.

Experimental protocol II: Motility and survival of worms

Synchronized (young) adult worms were transferred to control or test (standard NaCl concentration plus 100–700 mmol/L NaCl) plates (NGM plates, seeded with *E. coli* OP50 or *E. coli* HT115 expressing dsRNA), and transient (motility) or permanent immobility (survival) was tested at different points in time by applying gentle touch stimuli.

Experimental protocol III: Changes in body volume

Single synchronized (young) adult worms were incubated for 24 hours in wells of 24-well microtitre plates, containing standard NGM medium plus 400 mmol/L NaCl as well as 10 μ L *E. coli* OP50 suspension. Using a microscope and camcorder, short image sequences were taken from each worm, before (control, 0 h) and after transferring worms to the hyperosmotic condition (5, 20, and 60 min, 4 and 24 h). ImageJ was used for image processing. One picture of each sequence was selected and converted to 8-bit gray scale, followed by background subtraction and threshold implementation. Then, the area of the worm was evaluated. Percentage changes in area (body volume) were calculated by normalizing with respect to the area under control conditions (0 h).

Experimental protocol IV: DAF-16 translocation assay

Synchronized (young) adult worms of the TJ356 strain were incubated for 24 hours on control or test (standard NaCl concentration plus 200–700 mmol/L NaCl) plates (NGM plates, seeded with *E. coli* OP50 or *E. coli* HT115 expressing dsRNA). A final heat stress (37°C, 1 h) was applied in a heating cabinet to check for proper DAF-16 nuclear translocation. After hyperosmotic (or heat) incubation, fluorescence images of randomly chosen worms were taken, and the percentage of worms showing only cytoplasmic localization of DAF-16::GFP determined.

RNA-Seq analysis

Synchronized (young) adult worms, WT and *kgb-1* Δ , from six different cultures each were incubated for 3 h under control and/or test (standard NaCl concentration plus 400 mmol/L NaCl) conditions (20°C)

on NGM plates, seeded with *E. coli* OP50 (per condition and strain, two plates containing several hundred worms each). After incubation, animals were washed off the plates with sterile water of corresponding NaCl molarity (washing medium). After washing them two times with 1 ml washing medium to get rid of feeding bacteria, and adding RNAiso-G (Segentec, Borken, Germany), they were frozen in liquid nitrogen. After a multiple thermal disruption of worms (liquid nitrogen, 35°C), chloroform extraction on ice (10 min), and centrifugation (12,000 × g, 4°C, 15 min), RNA was isolated using RNase-free DNase sets and RNeasy® mini kits (Qiagen, Hilden, Germany). In addition, several provisions were made to work under RNase-free conditions. Quality control was carried out with an Agilent Bioanalyzer® (Agilent Technologies, Böblingen, Germany). After adding RNastable™ matrix (Biomatrica, San Diego, CA, USA), and vacuum centrifugation for drying (2 h), samples were sent to the Beijing Genomics Institute (BGI) for RNA-Seq analysis by Illumina HiSeq2000 technology. Briefly, mRNA was purified using oligo-(dT)-magnetic beads, and first and second strand cDNA-synthesis was carried out using random hexamer-primers. After ligation with sequencing adaptors, samples were sequenced with Illumina HiSeq2000 with minimally 10 Megareads and a sequencing quality of more than 98% clean reads. Reads were mapped to Wormbase release WS223. Gene expression was calculated with the RPKM-method (Reads Per Kb per Million reads) out of the number of reads for one gene, the transcript length, and the overall number of reads in the sample [35]. The *P*-value from differential gene expression tests [36] specifies the probability of a gene equally or unequally expressed between two samples, and the False Discovery Rate (FDR) is a method to determine the threshold of *P*-values in multiple tests. In accordance with the proposals of BGI, we took a FDR < 0.005 as threshold for differentially expressed genes.

Semi-quantitative RT-PCR

Synchronized (young) adult worms of wildtype and *kgb-1Δ* were washed off from control plates with washing medium (sterile water plus 51 mmol/L NaCl) and transferred to test (standard NaCl concentration plus 200, 300, or 400 mmol/L NaCl) plates (NGM plates, seeded with *E. coli* OP50). After incubation, worms were washed off and cleaned several times with washing medium to exclude bacteria. RNA was isolated using RNAiso-G. After reverse transcription of 1 µg total RNA per sample using oligo-(dT)₁₈ primers (First Strand cDNA Synthesis Kit; Fermentas, St. Leon-Rot, Germany), 1 µl cDNA was used for semi-quantitative RT-PCR. Using *cdc-42* as housekeeping gene [37] (primers: 5'-ATGCAGACGATCAAGTGGC-3', 5'-TTCAGTCCCTTCTGCGTCA-3'; reaction: 30 s at 94°C, 45 s at 53°C, 35 s at 72°C; 28 cycles), the relative expression levels of *kgb-1* (5'-GGAAGTGTCTGAATGGCCGATG-3', 5'-CGGCAAAATCAATTTCTTGATC-3'; 30 s at 94°C, 45 s at 61°C, 60 s at 72°C; 29 cycles), *jun-1a-e* (5'-ATGGCACTCGATGACCAAGA-3', 5'-CACGAATCGAATTGTTCTGGGC-3'; 30 s at 94°C, 45 s at 52°C, 15 s at 72°C; 30 cycles), and *fos-1* were determined. In case of *fos-1*, there are two overlapping splice variants (*fos-1a*, *fos-1b*), which cannot be clearly separated by PCR. Therefore, PCR was carried out for both *fos-1a* and *fos-1b* simultaneously (*fos-1ab*) (5'-TGGCGGAATGCAACAACATCCGA-3', 5'-GTCTGAATGGGCCTGGTGGGC-3'; 30 s at 94°C, 45 s at 55°C, 42 s at 72°C; 29 cycles). Quantification and analysis of band intensities were made using ImageJ.

GPDH activity assay

Synchronized (young) adult worms were incubated for different times (0–24 h) on test (standard NaCl concentration plus 200 mmol/L NaCl) plates (NGM plates, seeded with *E. coli* OP50). After incubation, worms were washed off and cleaned several times with washing medium to exclude bacteria. The samples were centrifuged (4°C, 1500 × g), and the supernatant removed. The worm pellet was weighed and estimating 1 mg as 1 µl, 1.5 volumes of lysis-buffer (100 mmol/L Glycine-NaOH pH 9.0, 5 mmol/L EDTA, 0.9 mol/L glycerol, 2 mmol/L PMSF) were added, with the samples then frozen in liquid nitrogen. After thawing on ice, samples were homogenized using a Teflon® pistil, centrifuged (4°C, 16000 × g) to remove insoluble material, and protein concentration of the supernatant determined using the Bradford assay. GPDH activity was determined via the reverse reaction: dihydroxyacetone phosphate + NAD⁺ → glycerol-3-phosphate (G3P) + NADH. For each measurement, 250 µl Borax solution (25 mmol/L) and 67 µl H₂O were pre-warmed to 30°C. Then, a 100 µl sample was added, followed by the addition of 50 µl NAD⁺ (50 mmol/L; after 3 min) and 33 µl G3P solution (150 mmol/L; after 6 min). The increase in NADH absorbance was measured at λ = 340 nm. The specific GPDH activity (nkat/mg protein) resulted from the slope of the NADH absorbance curve, with the extrapolated NAD⁺ baseline subtracted and the data normalized with respect to protein concentration.

Data bases

DEGs were classified according to WormBase (<http://www.wormbase.org/#01-23-6>) and KOG (Eukaryotic clusters of orthologous groups) (<http://www.ncbi.nlm.nih.gov/COG/>). Transport DB (www.membranetransport.org; [38]) was used to detect genes for membrane transporters in the transcriptome data.

Statistical analysis

Data are given as means $\pm$ standard deviation (S.D.) or means of means $\pm$ standard error of the mean (S.E.M.) with n indicating the number of analyzed plates or the number of experiments. Lifespan was determined with the Kaplan–Meier estimator, and statistical significances were checked using the logrank test. t-tests were applied to test for differences between control and/or test conditions, and one-way analysis of variance (ANOVA) with a subsequent multiple comparison procedure (Student Newman–Keuls method) was used to test for differences in time courses or NaCl concentration series. The chosen significance level was $P < 0.05$. Enrichment analyses (i.e., chi-square tests; Microsoft Excel 2007) were done to identify KOG categories with the ratio between up-regulated and total DEGs with KOG identity (KOG ID) deviating highly significantly ($P < 0.001$) or significantly ($P < 0.05$) from the corresponding overall ratio of the two evaluated contrasts (WT_{ho}/WT_{ctrl} : 448 vs 700 DEGs; $kgb-1\Delta_{ho}/WT_{ho}$: 801 vs 1171 DEGs). The gene functional classification tool (DAVID Bioinformatics Resources 6.7, <http://david.abcc.ncifcrf.gov/home.jsp>) was used to cluster all up- or down-regulated DEGs of the two contrasts (with simultaneous computation of group enrichment scores, GES). SigmaPlot 11.0 (Systat Software, Erkrath, Germany) was used for graph preparations and other statistical analyses.

Results

KGB-1 expression

KGB-1 expression was studied using transcriptional ($P_{kgb-1}::gfp$; Fig. 1A, B) and translational ($P_{kgb-1}-kgb-1::gfp$; Fig. 1C) reporters. Eggs already showed the corresponding GFP fluorescence (Fig. 1A), with more and more tissues following during development (L1 larval stage: pharynx, excretory cell, some sensory neurons; L2–L4 larval stages: intestine, rectal sensory neurons; adult worms: longitudinal muscles, gonads). The rare males showed GFP expression in their spermathecae. Within the anterior part of the nervous system, GFP expression was detected in the pharyngeal nerve ring and in some sensory neurons (Fig. 1B, a1–a3). Within its posterior part, lumbar ganglia and the dorso-rectal ganglion showed GFP expression (Fig. 1B, p).

Reproduction rate and lifespan at moderate hyperosmolarity

Moderate (i.e., non-lethal) hyperosmolarity (200 mmol/L NaCl) impaired reproduction in wildtype (WT) and several signaling mutants ($pmk-1\Delta$, $jnk-1\Delta$, $daf-2\Delta$), but not in $kgb-1\Delta$ (Fig. 2B). Under control conditions, however, the level of offspring was lower in $kgb-1\Delta$ (and $pmk-1\Delta$, $daf-2\Delta$) than in WT. $kgb-1\Delta$ and $daf-2\Delta$ also showed a time delay in egg-laying by one day at moderate hyperosmolarity (Fig. 2A). Moderate hyperosmolarity impaired lifespan in WT, $jnk-1\Delta$, and $daf-2\Delta$, but not in $kgb-1\Delta$ (Fig. 2C). However, the lifespan of $kgb-1\Delta$ was already short under control conditions. Hence, reproduction rate is low and lifespan short in $kgb-1\Delta$ but in both cases unaffected by moderate hyperosmolarity.

Survival rate at severe hyperosmolarity

Testing the tolerance to severe hyperosmolarity showed $kgb-1\Delta$ and $daf-2\Delta$ to survive 400 mmol/L NaCl and higher salt concentrations (24 hours) much better than WT, whereas the survival rates of $pmk-1\Delta$ and $jnk-1\Delta$ did not differ from WT (Fig. 3A). Another test series with 400 mmol/L NaCl (24 hours) confirmed the improved survival rate of $kgb-1\Delta$ and $daf-2\Delta$ in comparison to WT, whereas $pmk-1\Delta$, $jnk-1\Delta$, and the MAP2K mutants $mek-1\Delta$ and $sek-1\Delta$ did not survive hyperosmolarity better than WT (Fig. 3B). Similar results were obtained by RNAi experiments, which showed $kgb-1$ -RNAi to enhance the survival rate at 400 mmol/L

Fig. 1. KGB-1 expression. (A) The transcriptional reporter $P_{kgb-1}::gfp$ was expressed in eggs, L1 larvae (P: pharynx, E: excretory cell, SN: sensory neurons), from L2 stage onwards in intestinal cells (I) and rectal sensory neurons (rSN), and in longitudinal muscles (M) and mature gonads of adult worms (horizontal bars, 20 μ m). (B) $P_{kgb-1}::gfp$ was expressed in anterior (a1–a3) and posterior (p) parts of the nervous system (SN, SD: nuclei and dendrites of sensory neurons, NR: pharyngeal nerve ring, DRG: dorso-rectal ganglion, LG(r, l): right and left lumbar ganglia). (C) The translational reporter $P_{kgb-1}-kgb-1::gfp$ showed an expression pattern similar to that of the transcriptional reporter (P: pharynx, G: gonads).

Fig. 2. Reproduction and lifespan at moderate hyperosmolarity. (A) Daily or (B) total numbers of offspring per worm and (C) lifespan of WT, $kgb-1\Delta$, $pmk-1\Delta$, $jnk-1\Delta$, and $daf-2\Delta$ at control (white areas/bars) or hyperosmotic conditions (200 mmol/L NaCl; dotted areas/bars). (A, B: means $\pm$ S.D.; C: Kaplan-Meier curves; per condition and strain, $n = 8$ –13 plates with one worm each.) Asterisks indicate significant differences between control and hyperosmotic conditions; small letters (a, b) mark significant differences between WT and mutants either at control or hyperosmotic conditions ($P < 0.05$).

NaCl (24 hours), whereas *sek-1*-RNAi in WT or *mek-1* Δ did not improve hyperosmotic tolerance in comparison to the control- (ctrl-) RNAi treated equivalents (Fig. 3C). Thus, the knock-out (mutant) or knock-down (RNAi) of KGB-1 and DAF-2 expression promote the tolerance to severe hyperosmolarity.

Fig. 3. Survival at severe hyperosmolarity. (A) Survival rates of WT (dotted areas), *kgb-1* Δ , *pmk-1* Δ , *jnk-1* Δ , and *daf-2* Δ after 24 hours at different osmotic conditions (0–700 mmol/L NaCl). (B) Survival rates of WT, *kgb-1* Δ , *pmk-1* Δ , *jnk-1* Δ , *mek-1* Δ , *sek-1* Δ , and *daf-2* Δ after 24 hours at control (white bars) or hyperosmotic conditions (400 mmol/L NaCl; dotted bars). (C) Survival rates of RNAi-treated WT (ctrl-RNAi), *kgb-1*-RNAi, *sek-1*-RNAi, *kgb-1* Δ (ctrl-RNAi), and *mek-1* Δ (ctrl-RNAi, *sek-1*-RNAi) after 24 hours at control (white bars) or hyperosmotic conditions (400 mmol/L NaCl; dotted bars). (Means $\pm$ S.D.; per condition and strain/treatment, $n = 5$ –37 plates with 10 worms each.) Asterisks indicate significant differences between control and hyperosmotic conditions; small letters (*a*) or asterisks and bars mark significant differences between WT and mutants or different RNAi-treatments ($P < 0.05$).

Fig. 4. Body volume and motility at acute and severe hyperosmolarity. Changes in (A) body volume and (B) motility (normalized data; initial values = 100%) after transferring WT (dotted areas), *kgb-1* Δ , and *daf-2* Δ as well as (A) *mek-1* Δ or (B) *pmk-1* Δ and *jnk-1* Δ from control (time: 0 h) to hyperosmotic conditions (400 mmol/L NaCl). (Means $\pm$ S.D.; per strain, either (A) $n = 5$ –16 experiments on one worm each or (B) $n = 14$ –20 plates with 10 worms each.) Asterisks indicate significant differences between WT and mutants ($P < 0.05$).

Body volume and motility at acute and severe hyperosmolarity

Studying effects of acute and severe hyperosmolarity (400 mmol/L NaCl) on body volume and motility showed for both variables initial decreases (0–1 hour) and subsequent

Fig. 5. Glycerol-3-phosphate dehydrogenase (GPDH) activity at moderate hyperosmolarity. (A) Changes in GPDH activity after transferring WT (dotted area) and *kgb-1* Δ from control (time: 0 h) to hyperosmotic conditions (200 mmol/L NaCl). (B) GPDH activity after 24 hours at control (white bars) or hyperosmotic conditions (200 mmol/L NaCl; dotted bars) in WT, *kgb-1* Δ , *pmk-1* Δ , and *jnk-1* Δ . (Means $\pm$ S.E.M.; per condition and strain, $n = 3$ plates with several hundred worms each.) Asterisks indicate significant differences between control and hyperosmotic conditions; small letters (a) or asterisks and bars mark significant differences between WT and mutants ($P < 0.05$).

Fig. 6. Effects of KGB-1 and hyperosmolarity on *kgb-1*, *jun-1*, and *fos-1* expression. Levels of (A) *kgb-1* mRNA, (B, C) *jun-1a-e* mRNA, (D, E) *fos-1ab* mRNA and (F) ECFP fluorescence intensity from the expressed transcriptional reporter $P_{fos-1}::ecfp$ in (A, B, D, F) WT and (C, E) *kgb-1* Δ after three hours (mRNA) or three and 24 hours (protein) at hyperosmotic conditions (200, 300, and 400 mmol/L NaCl; all data sets normalized with respect to control conditions (= 100%). (G) P_{fos-1} -dependent ECFP expression in the posterior worm region after 24 hours at control or hyperosmotic conditions (200 mmol/L NaCl). (Means $\pm$ (A–E) S.E.M. or (F) S.D.; per condition and strain, either (A–E) $n = 3$ plates with several hundred worms each or (F) $n = 5$ –10 plates with one worm each.) Asterisks and bars indicate significant differences in expression level; small letters (a) mark significant differences between WT and *kgb-1* Δ ($P < 0.05$).

increases (0.5–24 hours) (Fig. 4). The return to initial values was faster in *kgb-1* Δ than in WT (Fig. 4A, B). The MAP2K mutant *mek-1* Δ showed only a small initial decrease in

Fig. 7. FOS-1 and hyperosmotic tolerance. Survival rates of (A) RNAi-treated WT (ctrl-RNAi, *kgb-1*-RNAi, *fos-1*-RNAi) and *kgb-1* Δ (ctrl-RNAi, *fos-1*-RNAi) or (B) WT and *fos-1* Δ after 24 hours at control (white bars) or hyperosmotic conditions (400 mmol/L NaCl; dotted bars). (C) Percentage of RNAi-treated (ctrl-RNAi, *kgb-1*-RNAi, *fos-1*-RNAi) DAF-16::GFP expressing worms (strain TJ356) with exclusively cytoplasmic DAF-16::GFP after 24 hours at control (white bars) or hyperosmotic conditions (400 mmol/L NaCl; dotted bars). (Means $\pm$ S.D.; per condition and strain/treatment, $n = 5$ –37 plates with 5–10 worms each.) Asterisks indicate significant differences between control and hyperosmotic conditions (C only); asterisks and bars mark significant differences between WT, mutant or different RNAi-treatments ($P < 0.05$).

body volume, and the recovery of body volume and/or motility was similar or inferior in *pmk-1* Δ , *jnk-1* Δ , and *daf-2* Δ in comparison to WT. Consequently, *kgb-1* Δ recovers faster than WT from the acute effects of severe hyperosmolarity on body volume and motility.

GPDH activity at moderate hyperosmolarity

Differences in hyperosmotic tolerance can be caused by differences in the activity of glycerol-3-phosphate dehydrogenase (GPDH), which is rate limiting for synthesis of the important compatible osmolyte glycerol. GPDH activity was determined upon acute and moderate hyperosmolarity (200 mmol/L NaCl). Similar to previously measured time courses of glycerol accumulation [39], GPDH activity increased in WT after approximately three hours reaching maximal levels after 18–24 hours (Fig. 5A). In *kgb-1* Δ , GPDH activity changed only slightly during this period. Another test series with 200 mmol/L NaCl (24 hours) showed a stronger increase in GPDH activity in WT (and *pmk-1* Δ) in comparison to *kgb-1* Δ (and *jnk-1* Δ) (Fig. 5B). Thus, the higher hyperosmotic tolerance of *kgb-1* Δ is not based on elevated GPDH activity and glycerol synthesis.

jun-1 and *fos-1* expression: effects of hyperosmolarity and *kgb-1* mutation

JNK promotes the expression of *jun-1* and *fos-1* [40], which code for AP-1 transcription factor elements [41]. Consequently, we checked whether *jun-1* and *fos-1* expression are likewise affected by the JNK-like MAPK KGB-1. In addition, we tested effects of different levels of hyperosmolarity (200, 300, 400 mmol/L NaCl). Rising hyperosmolarity caused the mRNA levels of *kgb-1*, *jun-1a-e*, and *fos-1ab* in WT to decrease (Fig. 6A, B, D) and of *jun-1a-e* and *fos-1ab* in *kgb-1* Δ to show a trend towards a decrease (Fig. 6C, E). Moderate hyperosmolarity,

Fig. 8. DAF-16 and hyperosmotic tolerance. (A) DAF-16::GFP cellular localizations in the transgenic strain TJ356 after 24 hours at control (ctrl; left) or hyperosmotic conditions (400 mmol/L NaCl; center) and in the control animals after one additional hour at 37°C (right). (B) Survival rates of WT (dotted area) and TJ356 (squares) as well as the percentage of worms with exclusively cytoplasmic DAF-16::GFP (triangles) after 24 hours at different

osmotic conditions (0–700 mmol/L NaCl). (C) Survival rates of RNAi-treated WT (ctrl-RNAi, *daf-16*-RNAi) and *kgb-1Δ* (ctrl-RNAi, *daf-16*-RNAi) after 24 hours at control (white bars) or hyperosmotic conditions (400 mmol/L NaCl; dotted bars). (Means $\pm$ S.D.; per condition and strain/treatment, $n = 3$ –37 plates with 5–10 worms each.) Asterisks indicate significant differences to the control condition (0 mmol/L NaCl) in DAF-16::GFP localization; small letters (a) or asterisks and bars mark significant differences in survival rate between WT and TJ356 or between different RNAi-treatments ($P < 0.05$).

however, often evoked higher expression levels in comparison to control values (normalized levels greater than 100%). In *kgb-1Δ*, mRNA levels (moderate hyperosmolarity) were lower (*jun-1a-e*; Fig. 6B, C) or tended to be lower (*fos-1ab*; Fig. 6D, E) than in WT. The significant decrease in P_{fos-1} -dependent protein (ECFP) expression with increasing hyperosmolarity (24-hour incubations; Fig. 6F, G) was in line with the decrease in *fos-1ab* mRNA expression (Fig. 6D). Thus, severe hyperosmolarity impairs the expression of *kgb-1*, *jun-1*, and *fos-1*, and KGB-1 (in WT) promotes the expression of *jun-1* and likely *fos-1*.

Hyperosmotic tolerance and FOS-1

fos-1-RNAi enhanced the hyperosmotic tolerance (400 mmol/L NaCl, 24 hours) of *C. elegans* even a little more than *kgb-1*-RNAi or *kgb-1Δ* (Fig. 7A), and *fos-1Δ* showed a higher hyperosmotic tolerance than WT (Fig. 7B). Moreover, *fos-1*-RNAi prevented, in contrast to control- and *kgb-1*-RNAi, nuclear DAF-16::GFP translocations in TJ356 (a DAF-16::GFP expressing transgenic strain) at severe hyperosmolarity (400 mmol/L NaCl, 24 hours) (Fig. 7C) indicating DAF-16 target (stress) gene expression not to be necessary in case of *fos-1*-RNAi. Blocked FOS-1 expression promotes the hyperosmotic tolerance of *C. elegans* even more than missing KGB-1, which is in line with the reported downward control of FOS-1 by KGB-1 [25, 28].

Hyperosmotic tolerance and the DAF-2 pathway

To assess contributions of the DAF-2 pathway to hyperosmotic tolerance, nuclear DAF-16::GFP translocations were studied in TJ356 together with survival rates of WT and TJ356 at different hyperosmotic conditions (24 hours; Fig. 8A, B). The DAF-16 overexpressing TJ356 (P_{daf-16} -*daf-16::gfp* [24]) survived higher salt concentrations (500–600 mmol/L NaCl) better than WT. Nuclear DAF-16::GFP translocations were detected at 400 mmol/L NaCl and higher salt concentrations (Fig. 8A, B), with the level of nuclear DAF-16::GFP decreasing again at 700 mmol/L NaCl. *daf-16*-RNAi affected the hyperosmotic tolerance of WT as well as *kgb-1Δ* negatively (Fig. 8C). Hence, the expression of DAF-16 target genes improves the tolerance to severe hyperosmolarity.

Fig. 9. KOG-classified DEGs in case of WT_{ho}/WT_{ctrl} and $kgb-1\Delta_{ho}/WT_{ho}$. Numbers of up- or down-regulated differentially expressed genes (DEGs; false discovery rate, FDR < 0.005) within the different KOG (eukaryotic orthologous groups) categories for (A) WT_{ho}/WT_{ctrl} and (B) $kgb-1\Delta_{ho}/WT_{ho}$. KOG categories with the ratio between up-regulated and total DEGs deviating highly significantly ($P < 0.001$) from the overall ratio between up-regulated and total DEGs are indicated by dark-gray bars (gray bars, $P < 0.05$).

Transcriptomics by RNA-Seq

To further elucidate transcriptional responses to severe hyperosmolarity (see [42] for responses to moderate hyperosmolarity) as well as KGB-1-dependent effects on gene expression, transcriptome profiling by RNA-Seq was carried out to contrast 1) severe hyperosmotic (ho ; 400 mmol/L NaCl, 3 hours) and control conditions ($ctrl$) in WT (WT_{ho}/WT_{ctrl}) and 2) $kgb-1\Delta$ and WT at severe hyperosmolarity ($kgb-1\Delta_{ho}/WT_{ho}$). In case of WT_{ho}/WT_{ctrl} , 700 differentially expressed genes (DEGs with a false discovery rate, FDR < 0.005) were detected (448 up-regulated and 252 down-regulated DEGs). For $kgb-1\Delta_{ho}/WT_{ho}$, 1171 DEGs were found (801 up-regulated and 370 down-regulated DEGs). Their functional classification according to KOG (eukaryotic orthologous groups) resulted for WT_{ho}/WT_{ctrl} in 457 DEGs with KOG identity (KOG ID) and for $kgb-1\Delta_{ho}/WT_{ho}$ in 610 DEGs with KOG ID. Thus, DEGs with KOG ID represented sufficiently large samples to classify them into higher-level KOG categories (Fig. 9). KOG enrichment analyses (i.e., chi-square tests) were carried out to identify KOG categories with the ratio between up-regulated and total DEGs deviating highly significantly ($P < 0.001$) from the corresponding overall ratio (WT_{ho}/WT_{ctrl} : 448 vs 700 DEGs; $kgb-1\Delta_{ho}/WT_{ho}$: 801 vs 1171 DEGs). In case of WT_{ho}/WT_{ctrl} , two KOG categories complied with this requirement ([W] Extracellular structures, $P = 3.5E-04$; [G] Carbohydrate transport and metabolism, $P = 2.0E-04$) (Fig. 9A; dark-gray bars). For $kgb-1\Delta_{ho}/WT_{ho}$, one category met this criterion ([J] Translation, ribosomal structure and biogenesis, $P = 3.9E-17$) (Fig. 9B; dark-gray bar).

Genetic inductions in WT and $kgb-1\Delta$ by severe hyperosmolarity

In case of WT_{ho}/WT_{ctrl} (Fig. 9A), the up-regulated genes of category [W] were mostly DEGs for type IV and XIII collagens (Fig. 10A) and in case of category [G], DEGs for UDP-glucuronosyl and UDP-glucosyl transferases (including one UDP-glucose pyrophosphorylase) as well as DEGs for enzymes involved in trehalose metabolism (*tps-2*, *tre-5*) and membrane transports (R10D12.1, *vglu-2*) (Fig. 10B). With respect to all up-regulated DEGs from WT_{ho}/WT_{ctrl} , the group enrichment score (GES; gene functional classification, DAVID 6.7) likewise

Fig. 10. DEGs of two KOG categories strongly deviating from standard regulation in case of WT_{ho}/WT_{ctrl} (see Fig. 9A). Log₂-fold changes in the expression of up-regulated DEGs within the KOG categories (A) [W] Extracellular structures (gray bars: type IV and XIII collagens) and (B) [G] Carbohydrate transport and metabolism (gray bars: UDP-glucuronosyl/-glucosyl transferases; dotted bars: genes for enzymes possibly involved in osmolyte synthesis and transport).

Fig. 11. DEGs of one KOG category strongly deviating from standard regulation in case of $kgb-1\Delta_{ho}/WT_{ho}$ (see Fig. 9B). Log₂-fold changes in the expression of down-regulated DEGs within the KOG category [J] Translation, ribosomal structure and biogenesis (white bars, different shadings: 60S, 40S, and mitochondrial ribosomal proteins; gray bars: translation factors; dark-gray bars: tRNA synthases).

turned out to be highest (GES = 6.6) for a cluster consisting only of genes for type IV and XIII collagens (24 genes in total).

In case of $kgb-1\Delta_{ho}/WT_{ho}$ (Fig. 9B), the down-regulated genes of category [J] comprised twenty DEGs for 60S ribosomal proteins, sixteen DEGs for 40S ribosomal proteins, and three DEGs for mitochondrial ribosomal proteins (commonly referred to as ribosomal protein genes, *RP* genes [43]) as well as ten DEGs for translation factors and five DEGs for tRNA synthases (commonly summarized as ribosome biogenesis genes, *Ribi* genes [43]) (Fig. 11). With respect to all down-regulated DEGs from $kgb-1\Delta_{ho}/WT_{ho}$, the GES was indeed highest (22.1) for a cluster consisting of genes for eighteen 60S ribosomal proteins, fourteen 40S ribosomal proteins, three mitochondrial ribosomal proteins, and only two other proteins. The categories [T] and [O] also contained many DEGs (Fig. 9B), including eleven DEGs for serine/threonine/(tyrosine) kinases, four DEGs for tyrosine kinases, two DEGs for protein phosphatase 1 regulatory subunits, eighteen DEGs for tyrosine- or serine/threonine-specific protein phosphatases, and seven DEGs for C-type lectins (category [T]) as well as three DEGs for antioxidant/redox enzymes, seventeen DEGs for chaperones, and eleven DEGs for enzymes involved in ubiquitination/proteasomal degradation (category [O]).

Table 1. DEGs in case of WT_{ho}/WT_{ctrl} (sequence, public gene name, function, $\log_2$ -fold change, regulatory direction) for membrane transports and osmolyte syntheses identified using WormBase^a and/or KOG^b, with asterisks indicating the respective false discovery rate (*: < 0.005; **: < 0.001)

Sequence	Public name	Function	$\log_2(WT_{ho}/WT_{ctrl})$	Regulation
Membrane transport				
R10D12.1		Permease of the major facilitator superfamily [KOG2532] ^b ; 86.8% homology to human vesicular glutamate transporter 2 ^a	2.8459	Up**
ZK550.2		Predicted transporter/transmembrane protein [KOG2325] ^b ; 91.6% homology to human major facilitator superfamily domain-containing protein 8 ^a	1.1917	Up**
K10G9.1	<i>vglu-2</i>	Vesicular glutamate transporter ^a	0.9313	Up**
Y32F6B.1		Organic anion transporter [KOG3626] ^b	0.8459	Up**
F26D10.9	<i>atgp-1</i>	Predicted amino acid transporter glycoprotein subunit ^a	0.7662	Up**
F35H10.4	<i>vha-5</i>	Ortholog of subunit a of the V0 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	0.4572	Up**
C46C2.2		Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily) [KOG0255] ^b	0.3887	Up**
T01H3.1	<i>vha-4</i>	Ortholog of subunit c ^a of the V0 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	0.3644	Up**
R10E11.8	<i>vha-1</i>	Ortholog of subunit c of the V0 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	0.3339	Up*
F20B6.2	<i>vha-12</i>	Ortholog of subunit B of the V1 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	0.1606	Up**
Y49A3A.2	<i>vha-13</i>	Ortholog of subunit A of the V1 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	0.1142	Up*
Y32F6A.4		Amino acid transporters [KOG1303] ^b	-0.9146	Down**
Osmolyte synthesis				
C23H3.7	<i>tre-5</i>	Trehalase ^a	4.0072	Up**
F47G4.3	<i>gpdh-1</i>	Glycerol-3-phosphate dehydrogenase ^a	1.9865	Up**
F19H8.1	<i>tps-2</i>	Trehalose-6-phosphate synthase ^a	0.7553	Up**

Table 2. DEGs in case of $kgb-1\Delta_{ho}/WT_{ho}$ (sequence, public gene name, function, $\log_2$ -fold change, regulatory direction) for membrane transports identified using WormBase^a and/or KOG^b, with asterisks indicating the respective false discovery rate (*: < 0.005; **: < 0.001)

Sequence	Public name	Function	$\log_2(kgb-1\Delta_{ho}/WT_{ho})$	Regulation
Membrane transport				
C43F9.6	<i>nkb-2</i>	Na ⁺ /K ⁺ -ATPase, beta subunit ^a	3.3518	Up**
Y46G5A.30	<i>snf-5</i>	Sodium-neurotransmitter symporter family ^a	2.9701	Up**
C01G12.8	<i>catp-4</i>	Cation transporting ATPase ^a ; Na ⁺ /K ⁺ -ATPase, alpha subunit [KOG0203] ^b	1.9698	Up**
Y51A2D.4	<i>hmit-1.1</i>	H ⁺ -dependent myo-inositol transporter ^a	1.3486	Up*
Y43F4B.7		Amino acid transporter [KOG1304] ^b	0.478	Up**
M02F4.8	<i>aqp-7</i>	Aquaglyceroporin ^a	0.2659	Up**
F52E1.10	<i>vha-18</i>	Ortholog of subunit H of the V1 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	0.2596	Up*
VW02B12L.1	<i>vha-6</i>	Ortholog of subunit a of the V0 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	0.2084	Up**
B0365.3	<i>eat-6</i>	Ortholog of the alpha subunit of a Na ⁺ /K ⁺ -ATPase ^a	0.0817	Up**
F20B6.2	<i>vha-12</i>	Ortholog of subunit B of the V1 domain of vacuolar proton-translocating ATPase (V-ATPase) ^a	-0.1884	Down**

DEGs for membrane transports and osmolyte syntheses

Supported by 'Transport DB' [38], we then analyzed the transcriptome data for potentially stress-relevant DEGs coding for membrane transporters. This search revealed twelve DEGs (eleven of them up-regulated) in case of WT_{ho}/WT_{ctrl} (Table 1) and ten DEGs (nine up-regulated) for $kgb-1\Delta_{ho}/WT_{ho}$ (Table 2). The two gene groups included three DEGs for members of the major facilitator family, one DEG for a glutamate transporter, and five DEGs for subunits of the V-ATPase, which particularly energizes apical membranes (WT_{ho}/WT_{ctrl}) as well as DEGs for one sodium-neurotransmitter symporter, one H⁺-dependent myo-inositol transporter, one aquaglyceroporin, and three DEGs each for V-ATPase subunits and subunits of the Na⁺/K⁺-ATPase, which energizes baso-lateral membranes ($kgb-1\Delta_{ho}/WT_{ho}$). In case of WT_{ho}/WT_{ctrl} , DEGs for enzymes involved in osmolyte synthesis were found in addition (Table 1).

Fig. 12. DEGs identified as germline-enriched genes in case of WT_{ho}/WT_{ctrl} and *kgb-1*Δ_{ho}/WT_{ho}. (A) In case of *kgb-1*Δ_{ho}/WT_{ho}, highly up-regulated genes for major sperm proteins (MSPs) and MSP domain proteins (MDPs; dotted bars) were detected. Identity to 'germline-enriched' genes [45] was found to be lower in case of (B) WT_{ho}/WT_{ctrl} than (C) *kgb-1*Δ_{ho}/WT_{ho} (94 vs 324 DEGs), with the latter contrast including 162 up-regulated (white bars) and no down-regulated (dotted bars) 'spermatogenesis-enriched' genes.

DEGs identified as DAF-16 target genes

To check for DAF-16 target gene expressions, the WT_{ho}/WT_{ctrl} and *kgb-1*Δ_{ho}/WT_{ho} data were analyzed with respect to 486 identified DAF-16 target genes [44] resulting in 46 (WT_{ho}/WT_{ctrl}) or 66 (*kgb-1*Δ_{ho}/WT_{ho}) hits. For WT_{ho}/WT_{ctrl}, the more highly up-regulated DEGs (log₂-fold changes ≥ 1) included one DEG for a small heat shock protein (*hsp-12.6*) and three DEGs for cytochrome P450s (*cyp-35C1*, *cyp-35A2*, *cyp-35A3*) as well as two yet not functionally described genes (*dod-3*, F13H6.3). In case of *kgb-1*Δ_{ho}/WT_{ho}, the more highly up-regulated DEGs coded for one MSP (*msp-31*), one ShK-like toxin peptide (T24B8.5), one C-type lectin (*clec-52*), and eight yet not functionally described gene products (C50F7.5, F35E12.5, T24E12.5, T02B5.1, T16G12.4, C05B5.2, F08G5.6, Y32H12A.8). No specific differences in up- and down-regulations were found for the twenty DAF-16 target genes identical in WT_{ho}/WT_{ctrl} and *kgb-1*Δ_{ho}/WT_{ho}.

DEGs identified as germline-enriched genes

The *kgb-1*Δ_{ho}/WT_{ho} transcriptome data contained many highly up-regulated DEGs for the spermatocyte-specific major sperm proteins (MSP) and MSP domain proteins (MDPs) (Fig. 12A). With respect to all up-regulated DEGs from *kgb-1*Δ_{ho}/WT_{ho}, the GES was indeed highest (9.0) for a cluster consisting of genes for 23 MSPs, 7 MDPs and only seven other proteins. Consequently, we analysed the WT_{ho}/WT_{ctrl} and *kgb-1*Δ_{ho}/WT_{ho} data with respect to 3143 germline-enriched genes [45], which had been subclassified into 1248 intrinsic genes (i.e., involved in mitotic proliferation and early meiosis I in the distal germline), 1030 oogenesis-

enriched genes (i.e., increased expression in hermaphrodites producing only oocytes), and 865 spermatogenesis-enriched genes (i.e., increased expression in hermaphrodites producing only sperm), with a possible overlap between intrinsic and oogenesis-enriched genes. 94 DEGs in case of WT_{ho}/WT_{ctrl} (Fig. 12B) and 324 DEGs for $kgb-1\Delta_{ho}/WT_{ho}$ (Fig. 12C) were identical to germline-enriched genes. Particularly striking was the high identity (18.7%) with spermatogenesis-enriched genes in case of $kgb-1\Delta_{ho}/WT_{ho}$ (162 up-regulated DEGs; Fig. 12C). Further specific investigation of the transcriptome data revealed *fog-1* (isoform b) and *fog-3*, whose protein products promote sperm fate specification [46, 47], as highly up-regulated DEGs ($\log_2$ -fold changes: 2.47 and 3.74) in case of $kgb-1\Delta_{ho}/WT_{ho}$. The germline RNA helicase genes *glh-1* and *glh-4* were also up-regulated DEGs ($\log_2$ -fold changes: 0.25 and 0.24) in case of $kgb-1\Delta_{ho}/WT_{ho}$. Thus, *kgb-1* mutation ($kgb-1\Delta_{ho}/WT_{ho}$) promotes spermatogenesis.

Discussion

Moderate hyperosmolarity did not significantly alter reproduction rate and lifespan in *kgb-1* Δ (Fig. 2), and *kgb-1* mutation/RNAi clearly promoted the tolerance to severe hyperosmolarity (Fig. 3, 4). In agreement with a previous study [29], KGB-1 activity (in WT) reduced the stress resistance of the (young) adult worms used in our study. Expression of *gpdh-1* and GPDH activity, which serve as standard osmoprotection the synthesis of glycerol [3], were however higher in WT than in *kgb-1* Δ (Table 1; Fig. 5). In line with the downstream control of AP-1 genes (*jun*, *fos*; Fig. 6) and elements [25, 28] by KGB-1, *fos-1* Δ /RNAi promoted hyperosmotic tolerance even more than *kgb-1* Δ /RNAi (Fig. 7). The low reproduction rate and shortened lifespan of *kgb-1* Δ (Fig. 2) went hand in hand with a reduced expression of genes for protein biosynthesis (Fig. 9B, 11). Genes for spermatogenesis, however, were highly up-regulated in *kgb-1* Δ (Fig. 12). Thus, KGB-1 (in WT) evidently promotes reproduction, lifespan, and protein biosynthesis and also ensures balanced gametogenesis but affects hyperosmotic tolerance negatively, even though a standard osmoprotection (glycerol accumulation) was more active when KGB-1 was operative. These basic facts will now be examined in detail.

KGB-1, protein biosynthesis and chaperones, reproduction and lifespan

KGB-1 promotes reproduction and lifespan in WT (Fig. 2) (see also [28, 29]). Genes for translation, ribosomal structure and biogenesis (KOG category [J]) were severely down-regulated in case of $kgb-1\Delta_{ho}/WT_{ho}$ (Fig. 9b) and predominantly up-regulated in case of WT_{ho}/WT_{ctrl} (Fig. 9a). It can therefore be concluded that the down-regulation of *RP* and *Ribi* genes (Fig. 11) was due to *kgb-1* mutation and not hyperosmolarity. Reduced translation rates (down-regulated *RP* and *Ribi* genes) could impair reproduction and may also shorten lifespan, when developmental disorders occur due to inadequate protein provision. The advantage of a lowered protein biosynthesis rate would be a reduced risk of overloading the chaperone machinery and producing misfolded proteins under stress [6], which could be a key mechanism for the enhanced hyperosmotic tolerance of *kgb-1* Δ (see below). Actually, *gpdh-1* expression, GPDH activity, and glycerol accumulation have been shown to be promoted by disturbed protein homeostasis and enhanced protein damage [1, 3]. As this may happen more easily in WT, the up-regulated *gpdh-1* expression and GPDH activity in this strain (Table 1; Fig. 5) may result from an overstressed chaperone machinery. The down-regulation of seventeen DEGs for chaperones and eleven DEGs for enzymes involved in ubiquitination/proteasomal degradation in case of $kgb-1\Delta_{ho}/WT_{ho}$ (see Results section) would also fit in the scenario of an under-utilized chaperone machinery in *kgb-1* Δ and less protein damage in *kgb-1* Δ than in WT at severe hyperosmotic stress (see below). The opposite effect of a KGB-1-mediated activation of the ubiquitin proteasome system has been reported by [28].

RP and *Ribi* genes are tightly co-regulated genes in response to varying cellular conditions [43, 48, 49], and *RP* genes have shared promoter motifs also in *C. elegans* [50]. Ribosome

biogenesis, translation, transcription, metabolism, and autophagy are controlled by the TOR pathway [51, 52], with a TORC1 substrate (Sch9 kinase; [53]) functioning as master regulator of ribosome biogenesis [54]. JNK activates mTORC1 signaling by phosphorylation of the mTORC1 protein Raptor [55]. As JNK-1 and JKK-1 (the associated MAP2K) are expressed in *C. elegans* only in neurons [9, 10], whereas the JNK-like MAPK KGB-1 is expressed ubiquitously including non-neuronal cells (Fig. 1) (see also [28]), it is possible that KGB-1 promotes the expression of *RP* and *Ribi* genes via the TOR pathway.

Already known KGB-1 functions are interactions with germline RNA helicases (GLHs), which are constitutive elements of germline-specific P granules (i.e., germline determinants) [19, 56]. *kgb-1Δ* was reported to show a strongly up-regulated GLH-1 level and disorganized GLH-1 locations in germ cells [16]. It was also suggested that KGB-1 and CSN-5 control germline homeostasis, with KGB-1 targeting GLH-1 for proteasomal degradation. Germline RNA helicase genes (*glh-1*, *glh-4*; see Results section), spermatogenesis-enriched genes, and genes for the spermatocyte-specific MSPs and MDPs [57], which are dominant sperm proteins [58] that enable sperm motility [59] and affect as hormones female germ cells [60], were up-regulated in *kgb-1Δ* (*kgb-1Δ_{ho}*/WT_{ho}) but not in WT (WT_{ho}/WT_{ctrl}) (Fig. 12). The germline-specific genes *fog-1* and *fog-3* (feminization of germline; [61]) but not *tra-1*, whose protein product (TRA-1A) exercises transcriptional control over *fog-3* [62] and likely *fog-1* [63], were also highly up-regulated in *kgb-1Δ* (see Results section). FOG-1 and FOG-3 promote sperm fate specification [46, 47], and the phosphorylation state of FOG-3 regulates the initiation (unphosphorylated FOG-3) and maintenance (phosphorylated FOG-3) of the sperm fate program [61]. The specific kinases, which affect the phosphorylation state of FOG-3, are yet not known. In case KGB-1 would be such a FOG-3 phosphorylating kinase, the sperm fate program might be initiated in *kgb-1Δ*. Anyhow, the up-regulation of *fog-1* and *fog-3* definitely fits to the shift to spermatogenesis in *kgb-1Δ*. A previous study already reported a temperature-sensitive (> 25°C), sterile phenotype of *kgb-1Δ* [19]. The sterile *kgb-1Δ* worms produced sperm during the L4 stage but no functional oocytes. Thus, the reduced reproduction rate (Fig. 2) as well as the switch to spermatogenesis (Fig. 12) in *kgb-1Δ* at 20°C may be linked to a pre-stage of the sterile phenotype of *kgb-1Δ* at 25°C, even if negative effects of a reduced protein biosynthesis rate on the reproduction rate of the mutant would be considered in addition (see above).

Relationships between KGB-1, Jun-1 and Fos-1

Jun and Fos are elements of the activator protein 1 (AP-1) early response transcription factor [41]. The MAPK JNK activates c-Jun, which typically causes *c-jun* expression via binding of c-Jun:c-Fos, for example, to CRE-binding sites in the *c-jun* promoter. JNK additionally promotes *c-fos* expression by activating the transcription factor Elk-1, which can bind to ETS-binding sites in the regulatory region of *c-fos* [40]. Thus, Jun and Fos genes are potential downstream targets of the JNK-like MAPK KGB-1, which is supported by the existence of CRE-binding sites in the promoters of *jun-1c* and particularly *jun-1d* and ETS-binding sites in the regulatory regions of *fos-1a* and *fos-1b* from *C. elegans* (R. J. Paul, unpublished data). Previous studies already reported KGB-1-dependent *jun-1* expression [27] and JNK-1-mediated phosphorylation of c-Elk1 [64] in *C. elegans*. Another study showed *fos-1a* expression to be restricted to the somatic gonad, but *fos-1b* expression to occur in many cells and tissues of *C. elegans* [30]. This study showed positive effects of moderate hyperosmolarity (200 mmol/L NaCl) and negative effects of severe hyperosmolarity (400 mmol/L NaCl) on *kgb-1*, *jun-1a-e*, and *fos-1ab* transcription and *P_{fos-1}*-dependent ECFP expression (Fig. 6A, B, D, F). The significantly (or noticeably) lower expression of *jun-1a-e* (or *fos-1ab*) in *kgb-1Δ* than in WT at moderate hyperosmolarity shows that KGB-1 affects *jun-1a-e* (and likely *fos-1ab*) expression positively (Fig. 6B–E). The even higher hyperosmotic tolerance of *C. elegans* after *fos-1*-RNAi treatment in comparison to *kgb-1*-RNAi (Fig. 7A, C) is also in line with a downward control of *fos-1* expression and/or FOS-1 activation [25, 28] by KGB-1.

The dependence of *kgb-1*, *jun-1*, and *fos-1* expression on salt concentration in WT (Fig. 6A, B, D, F) implies a reciprocal relationship between the KGB-1/AP-1 signaling system and

hyperosmolarity, with its absence or weakness in *kgb-1Δ* (Fig. 6C, E) indicating that this relationship is established via KGB-1. Considering in addition the positive effects of KGB-1 on protein biosyntheses (Fig. 9B, 11), a possible stress tolerance mechanism emerges based on the reciprocal relationship between stress intensity and the KGB-1/AP-1 signaling system together with positive effects of KGB-1/AP-1 signaling on protein biosyntheses: moderate hyperosmolarity should promote protein biosyntheses via enhanced KGB-1/AP-1 signaling, which will probably also lead to an enlargement of the chaperone machinery (see [12] for KGB-1 impacts on ER stress management), whereas severe hyperosmolarity likely impairs protein biosyntheses via inhibited KGB-1/AP-1 signaling resulting in an easing the burden of the existing chaperone machinery not expandable under stress. Under all conditions, the balance between protein synthesis rate and the existing stock of chaperones must be maintained. In this framework, *kgb-1* mutation would impede the signal flow between stressor (hyperosmolarity) and the downward signaling system (AP-1; Fig. 6C, E), but enhance stress tolerance in this way by the resulting decrease in protein biosynthesis rate, which should lower the risk of overloading the existing chaperone machinery. Likewise, a recent paper has concluded that 'inhibition of protein translation protects extant proteins from damage brought about by an environmental stressor' [6].

Hyperosmotic tolerance mechanisms in WT

To intensify compatible osmolyte (glycerol) production, *gpdh-1* expression and GPDH activity were up-regulated in WT (Table 1; Fig. 5). Concerning another compatible osmolyte (trehalose), the elevated expression of *tre-5* (trehalase) and *tps-2* (trehalose-6-phosphate synthase 2) is however ambiguous. Whereas trehalase degrades trehalose to glucose, TPS1 and TPS2 activities (also referred to as trehalose-phosphatase, TPP; [65]) serve the production of trehalose from glucose-6-phosphate and UDP-glucose [66], with *tps-2* (TPS2) expression apparently more important for the hyperosmotic tolerance of *C. elegans* [4]. The much higher up-regulation of *tre-5* in comparison to *tps-2* indicates a shift from trehalose synthesis to trehalose degradation possibly to support glycolytic energy production during the acute and severe hyperosmotic stress (400 mmol/L NaCl) applied in the transcriptome study. Aside from syntheses, osmolyte imports (possibly energized by apical V-ATPases) by members of the major facilitator family, which transport a wide range of substrates (e.g., ions, sugars, amino acids; [67]), and/or a transporter (*vglu-2*) for the import of glutamate as compatible osmolyte [68] may contribute to the hyperosmotic tolerance of WT (Table 1).

Further genes up-regulated in WT under hyperosmotic conditions included genes for type IV and XIII collagens (Fig. 10A). The collagen genes mostly code for components of the cuticular exoskeleton [69], with their up-regulation possibly reducing the cuticle's water permeability. Two other up-regulated collagen genes (*emb-9*, *let-2*) code for components of the tissue-surrounding basement membranes [70]. Mutations in two of the up-regulated genes (*dpy-2*, *dpy-7*) or in another set of genes (e.g., *osr-1*, *osm-7*, *osm-11*), whose products are likely secreted from the hypodermis, affect the osmotic resistance of *C. elegans* positively [71, 72], which was explained by intensified glycerol synthesis due to specific cuticle defects (*dpy* mutants) or constitutive osmotic stress signaling (e.g., *osr* and *osm* mutants). UDP-glucuronosyl/-glucosyl transferases are involved in the export of toxic or reactive compounds [73], with their up-regulation (Fig. 10B) indicating peroxidized lipids and/or other cellular damage in WT at severe hyperosmolarity.

Hyperosmotic tolerance mechanisms in *kgb-1Δ*

Acute and severe hyperosmolarity resulted in transhypodermal water loss, reduced body volume, and severely restricted motility (Fig. 4), with the latter likely due to a decrease in turgor pressure and a weakening of the hydroskeleton. The faster recovery of *kgb-1Δ* in comparison to WT points to differences between both strains in the presence or strength of an immediately effective restore mechanism. It also implies a reduced risk of cell damage by molecular crowding [74] and elevated cellular ionic strength [1] in *kgb-1Δ*, which likely also contributed to its higher hyperosmotic tolerance. Regulatory volume increase (RVI;

[75]) is a good candidate for an immediately effective restore mechanism, which is based on the net import of inorganic ions to promote water reflux and cell volume recovery, and the subsequent exchange of inorganic ions against compatible osmolytes (e.g., glycerol, trehalose) that do not interfere with cell functions [4]. The distinct up-regulation of genes for membrane transporters in *kgb-1Δ*, which included genes for Na⁺/K⁺-ATPase α/β-subunits, aquaglyceroporin, a myo-inositol transporter, and a sodium-neurotransmitter symporter (*snf-5*) (Table 2), indicates a particular significance of RVI in this strain. The up-regulation also implies negative effects of KGB-1 (in WT) on gene expressions for membrane transporters, perhaps to reduce energy-consuming membrane transports under non-stress conditions. A previous study on transcriptional responses to acute hyperosmolarity [76] actually classified genes for β1-Na⁺/K⁺-ATPase and a sodium/myo-inositol transporter (SMIT) as early genes and that for aldose reductase (AR), which catalyzes synthesis of the compatible osmolyte sorbitol, as late gene. Other studies also showed an up-regulation of Na⁺-K⁺-ATPase α/β-subunits (mRNA, protein) at severe hyperosmolarity [77, 78]. As key element for the energization of membrane transports, a higher number of expressed Na⁺/K⁺-ATPases supports the import of inorganic ions/water and compatible osmolytes, resulting in an accelerated water uptake, a faster recovery of the hydroskeleton, and a shorter period of molecular crowding and elevated ionic strength. Up-regulated aquaglyceroporins (*aqp-7*; [79]) promote water and glycerol imports, and the up-regulated H⁺-dependent myo-inositol transporter (*hmit-1.1*) possibly uses the transmembranous proton gradient provided by V-ATPases for imports of myo-inositol (i.e., another compatible osmolyte). Actually, the hyperosmotic up-regulation of *hmit-1.1* as well as osmoprotection by intestinal HMIT-1.1 have already been shown in *C. elegans* [5]. The highly up-regulated *snf-5* has 31% identity to human *glyt1* (*slc6a9*), which codes for a transport protein (mammalian Na⁺- and Cl⁻-dependent glycine neurotransmitter transporter, GLYT1) involved in cell volume control and osmoprotection [80].

Hyperosmotic tolerance and the DAF-2 pathway

daf-2 mutation (resulting in a higher nuclear DAF-16 level) or the overexpression of DAF-16 in TJ356 [24, 44] improved (Fig. 8B) but *daf-16*-RNAi reduced (Fig. 8C) the hyperosmotic tolerance of *C. elegans*. Moreover, the hyperosmotic range was similar for nuclear DAF-16::GFP localization and the survival advantage of TJ356 in comparison to WT (Fig. 8B). Thus, the translocation of DAF-16 into cell nuclei and subsequent DAF-16 target gene expressions promote the tolerance to severe hyperosmolarity. A previous study already reported a DAF-16-mediated increase in hyperosmotic tolerance [4], which was related, inter alia, to genes for enzymes involved in trehalose synthesis (*tps-1*, *tps-2*; see above). However, nuclear DAF-16::GFP translocations were not detected due to the moderate stress applied in the previous study (200 mmol/L NaCl). Checking for DAF-16 target genes [44] revealed only one small heat shock protein (*hsp-12.6*) and three genes for cytochrome P450s, which may be involved in the solubilization of toxic compounds [73], as highly up-regulated DEGs in case of WT_{ho}/WT_{ctrl}. However, the incubation time for RNA-Seq (3 hours) was possibly too short to detect more up-regulated DAF-16 target genes as nuclear DAF-16 translocation was measured only after 24 hours of hyperosmotic incubation. In case of *kgb-1Δ*_{ho}/WT_{ho}, the elevated hyperosmotic tolerance of *kgb-1Δ* may additionally have lowered the demand for up-regulated DAF-16-mediated stress genes.

Conclusions

This study provided evidence for positive effects of KGB-1 on reproduction, lifespan, protein biosynthesis (*RP* and *Ribi* genes), and balanced gametogenesis, which seem to be affected, at least partly, by the KGB-1/AP-1 signaling line. KGB-1 mutation/RNAi enhanced the tolerance of *C. elegans* to hyperosmotic stress. The reciprocal relationship between stress intensity and KGB-1/AP-1 signaling and the positive relationship between KGB-1 signaling

and protein biosynthesis indicates a stress tolerance mechanism aiming at the maintained balance between protein syntheses and the stock of chaperones: moderate stress promotes protein syntheses including those for chaperones, whereas severe stress relieves the existing chaperone machinery by depressing protein syntheses. Thus, KGB-1 mutation/RNAi likely enhances hyperosmotic tolerance by a reduced protein biosynthesis rate together with derepressed gene expressions for membrane transporters, which may serve in WT for energy saving purposes. DAF-16 target gene expressions were found to contribute to hyperosmotic tolerance in addition but specific interactions with KGB-1 were not detected.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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