Linearly changing stress environment causes cellular growth phenotype

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Abstract

A hallmark of cellular stress response pathways is to protect cells from different environmental insults. While much is known about how cells are protected against acute stresses of different types, the mechanisms underpinning how cells are protected against gradually changing stresses are poorly understood. Here we demonstrate that a linear stress gradient but not a step, pulse or a quadratic gradient of the same stressor and intensity causes a severe cell growth phenotype. We determined that this phenotype is caused by the failure of cells to activate the stress-activated protein kinase signaling pathway due to a threshold in the rate of stress application. This lack of response occurs despite cells physically detecting the relatively slow changes in stress signal. These findings have fundamental implications for understanding mechanisms of how temporally changing environments impact biological phenotype.

Introduction

A hallmark of life is the ability of organisms to temporally sense and control their internal environment and temporally respond to external changes through the use of dedicated signal transduction pathways [1]. Some examples of the physiological importance of response systems include the temporal dynamics of hormone secretions [2], temporally regulated morphogen gradients [3], temporal dynamics in chemotaxis [4, 5], changes to bacterial and chemical cellular stresses [6] or osmolyte concentration change in the kidney [7]. Dysregulation of these mechanisms can cause imbalance in tissue homeostasis and contribute to human diseases [7, 8]. Despite the well characterized biological responses noted above, our understanding of how cells sense, process and respond to extracellular stress is based on experiments with acute or step-like changes of different intensities. In these types of experiments, the underlying assumption is that rapid environmental changes are physiologically representative. Although such experimental paradigms have proven critical in biological research by enabling the initial discovery of relevant biological molecules, pathways and mechanisms, they likely do not accurately reflect true physiological dynamics, and consequently neglect important temporal aspects of the biological process of interest [9, 10]. In this study, we systematically assessed how temporally changing stress environments affected cellular response at the molecular and cellular level. We utilized the well characterized High Osmolarity Glycerol (HOG) Stress Activated Protein Kinase (SAPK) signaling pathway in the yeast *Saccharomyces cerevisiae* model system (Fig. 1A) [11, 12]. This SAPK pathway is evolutionarily conserved from yeast to human (Hog1/p38) and is involved in many cellular and disease processes [13]. Upon rapid changes in external osmolarity, cell volume decreases, which induces phosphorylation and nuclear translocation of the Hog1 kinase (Figs. 2, S6) [14]. Phospho - Hog1 kinase cause changes in gene expression of many genes (including *STL1*, *GPD1*, and *GPP2*) [15] and accumulation of internal glycerol due to glycerol exporter deactivation [16] and increased glycerol production [12, 17]. Internal glycerol accumulation balances the external osmotic pressure and results in deactivation and nuclear export of the Hog1 kinase within a short time frame (Figs. 2C, S5, S6) [14]. YFP tagging of the Hog1 kinase allows its nuclear translocation to be monitored in real-time in live cells (Hog1 nuclear localization), and serves as a proxy for Hog1 phosphorylation dynamics. A major advantage of the yeast Hog1 pathway is that it enables highly accurate and reproducible single cell level quantification of the effects of temporal changes in the external environment on

signal detection (cell volume change), signal transduction (Hog1 nuclear localization), specific RNA gene transcription, and cellular growth.

Results

To answer the question of how different temporal changes in the environment affect cellular phenotype, we expose wild type (WT) *BY4741* cells to several osmotic stress challenges (Fig. 1B, S1-3, Table S3) and measured the cell population doubling time (Fig. 1C). In these experiments, cells were exposed to different temporal osmolyte profiles for 25 minutes and then the cells are exposed to media without osmolyte for the same amount of time in a periodic manner. The duration of 25 minutes was chosen because upon exposure of cells to an osmolyte step and pulse, the Hog1 kinase were transiently localizied to the nucleus within this time (Fig. 2C). The final concentration of 0.2M NaCl was chosen because it was identified previously as a mild stress to cells [18]. Using untreated cells as a control, we observe an increase in cell doubling time by ~2.5-fold when cells are exposed to repetitive linear ramps (magenta) to a final external osmolyte concentration of 0.2M NaCl (Fig. 1C). In contrast, when cells are exposed to a step (cyan), a single pulse (yellow), a repetitive series of pulses (black), or a series of quadratic ramps (green) of the same frequency and strength, only minimal increases in the cell doubling time were observed.

To understand the molecular mechanism for this unexpected slow growth phenotype upon a linear ramp profile, we exposed cells to different temporal osmolyte profiles with the same duration and final NaCl concentration (Fig. 2A) and quantified both changes in cell volume and Hog1 kinase signaling in single cells (Fig. 2B, C). We observed that all stresses result in a volume decrease due to an increase in external osmotic pressure in comparison to control media, indicating that all cells undergo osmolyte change in these environments (Fig. 2B). We then monitored Hog1 nuclear localization and observed that a linear ramp of 8mM NaCl per minute for 25 minutes (magenta) does not cause Hog1 nuclear localization (Fig. 2C). In comparison, an osmotic step (cyan) or quadratic ramp (green) (Fig. 2C, insert) with the same final osmolyte concentration and exposure time results in increased Hog1 nuclear localization (Figs. 2C; S6, Table S2). We hypothesize that the inability to activate Hog1 signaling should result in a reduction or elimination of Hog1-dependent stress response transcription activation.

To test this hypothesis, we exposed WT cells to a linear ramp of 8mM NaCl per minute (magenta) and a 0.2M NaCl step (cyan) (Fig. 3). We also exposed the Hog1 gene deletion strain (Hog1Δ) to a 0.2M NaCl step (black). We performed single molecule RNA FISH in individual cells on three stress response genes: *GPD1*, an NAD-dependent glycerol-3-phosphate dehydrogenase (GAPDH homolog), *GPP2*, a DL-glycerol-3-phosphate phosphatase and *STL1*, a glycerol proton symporter [15, 19] (Fig. 3A). All three genes are involved in glycerol biosynthesis and transport, and are therefore critically important for yeast cells adaptation of osmotic pressure changes [12]. We observed that the linear ramp in WT cells or a step of 0.2M NaCl in the Hog1Δ strain, at best only poorly activate *GPD1*, *GPP2*, and *STL1* transcription in comparison to a 0.2M NaCl step in WT cells (Figs. 3A, S4, and Table S1). These results indicate that a lack of Hog1 phosphorylation and nuclear localization result in significant reduction in mRNA transcription. Next, we asked whether, as predicted, the lack of Hog1 signaling and mRNA transcription activation result in reduced production of glycerol, the cellular osmolyte that protects yeast cells from osmotic stress (Figs. 3B, S5). We quantified cell internal glycerol concentrations in WT (cyan) and Hog1Δ (black) cells exposed to a step of 0.2M NaCl, WT cells exposed to a linear ramp of 8mM NaCl per minute (magenta) and WT cells exposed to a quadratic ramp to 0.2M NaCl for 25 minutes (green). We observed that cells exposed to a linear ramp have the lowest cell internal glycerol concentration, a value similar to Hog1Δ cells exposed to a 0.2M NaCl step. In comparison, cells exposed to a quadratic ramp contain more internal glycerol, which is consistent with an increase in Hog1 signaling after 20 minutes (Fig. 2C, insert) and a reduced doubling time (Fig. 1C). Based on these results, we hypothesize that the mechanism for Hog1 kinase activation is a threshold rate of osmolyte change to protect cells from a slowly changing environmental stress. Instead of performing many experiments with different osmolyte ramp rates to identify this threshold (Th) in the osmolyte rate, we utilized a quadratic osmolyte gradient with a final concentration of 0.2M NaCl (Fig. 2C, insert). In such an experiment, the osmolyte ramp rate changes linearly over time, and we can therefore sample many ramp rates to determine a threshold. To demonstrate the feasibility of this approach, we plotted Hog1 nuclear localization (Fig. 2C, green) as a function of the first derivative of the quadratic NaCl profile (rate of NaCl change) (Fig. 3C). We quantified that the Hog1 kinase is robustly activated after the NaCl ramp rate exceeds 13 ± 0.3mM NaCl per minute, demonstrating a clear threshold rate for Hog1 nuclear localization

(Figs. 2C insert, 3B). This result is supported by experiments in which cells are exposed to a constant osmolyte rate of 12mM NaCl per minute, which is just below the identified threshold, and does not result in Hog1 nuclear localization (Fig. S7).

To demonstrate that this behavior is primarily mediated through the Hog1 kinase, we transformed cells with a plasmid carrying the hyperactive Hog1 (HA-HOG1) allele under the control of the methionine repressive MET3 promotor [20, 21]. HA-Hog1 is a constitutively active variant of Hog1 that has full kinase activity without osmotic stress. Because HA-Hog1 can be reversibly induced upon a transient change in cell media by removing methionine, we hypothesized that transient activation of HA-Hog1 before osmotic stress would induce glycerol production and protect cells from harmful stress gradients (Fig. 4). To test this hypothesis, we induced HA-Hog1 20 minutes prior to applying a linear osmolyte gradient (Fig. 4A). We quantified the relative volume change in cells that are exposed to media (black) or cells that are exposed to a linear ramp rate of 8mM NaCl per minute for 25 minutes with (green) or without (magenta) prior transient activation of HA-Hog1 (Fig. 4B). In cells with HA-Hog1 induction (green), the cell volume change is similar to non-stress conditions (black) while non-induced HA-Hog1 cells exposed to the same linear osmolyte gradient (magenta) behave like WT cells subjected to the same salt stress (Fig. 2B). We then quantified internal glycerol concentrations in cells exposed to a linear ramp rate of 8mM NaCl per minute for 25 minutes with (green), or without (magenta) prior transient activation of HA-Hog1. These glycerol concentrations were compared to the internal glycerol concentrations in cells exposed to a step of 0.2M NaCl for 25 minutes without (black) prior transient activation of HA-Hog1. We observed that the transient activation of HA-Hog1 restores glycerol levels to levels similar to cells exposed to step of 0.2M NaCl (Fig. 4C). In addition, we observed that when cells are exposed to a series of ramps of 8mM NaCl per minute, the relative cell doubling time is reduced when HA-Hog1 was transiently activated 20 minutes before the ramp series (green) in comparison to cells without prior induction of HA-Hog1 (magenta) (Figs. 4D, S5). These results demonstrate that transient expression of HA-Hog1 alone is able to restore cell volume change, glycerol production and growth deficit and therefore reverse these cellular phenotypes.

Discussion

It has been shown previously that different temporal patterns of stresses can be used to probe signaling pathways to identify pathway models [14, 18], to probe pathway dynamic properties [22], to hyperactivate a pathway [23] or to assign biological function to pathway branches [24, 25]. In all of these cases rapidly changing environments activate Hog1 kinase robustly, resulting in strong gene expression response and an increase in glycerol levels that enable the cells to survive extracellular stress (Figure 4E, left panel). Here we discovered that if cells are exposed to a slowly changing linear stress gradient of the same type and the same final osmolyte concentration, cells still detect this stress as measured by reduced cell volume but are unable to phosphorylate and hence translocate Hog1 kinase to the nucleus. The consequence is a lack of mRNA transcription in single cells for several genes and a subsequent reduction of cell intracellular glycerol. As a mechanism, we identified a threshold rate of external osmolyte change that needs to be crossed to activate Hog1 phosphorylation and nuclear translocation, mRNA transcription activation and glycerol production (Figure 4E, middle panel). The observed phenotypes of altered volume change, glycerol production and reduced growth rate can be rescued through transient activation of HA-Hog1, which rescues the phenotype caused by linearly changing osmolyte concentration (Figure 4E, right panel).

Our results have general implications in many other organisms including how different temporal stress and environmental changes can dictate cellular phenotype. Because many signaling pathways in mammalian cells demonstrate adaptive behavior, it is likely that slowly changing environments are essential physiologically to the cell but may not be detected by a cell in a disease state. Alternatively, a slowly changing growth signal might, under normal conditions fail to activate proliferation, but in a disease state may activate uncontrolled cell growth contributing to cancer. If a threshold rate has been identified in a disease cell as the disease-causing mechanism, one could imagine developing drug intervention regimes that target the threshold rate. These inhibitors might be highly selective because they would function only under certain environmental conditions and otherwise have minimal potency. Our methodology utilizing different temporal profiles of extracellular molecules combined with traditional biological approaches demonstrate the power of dynamic perturbations for understanding biological mechanism and function that perfectly complement chemical biology and genetic

approaches. We therefore believe our work has far reaching applications to many other dynamic signal transduction and gene regulatory pathways.

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Figures

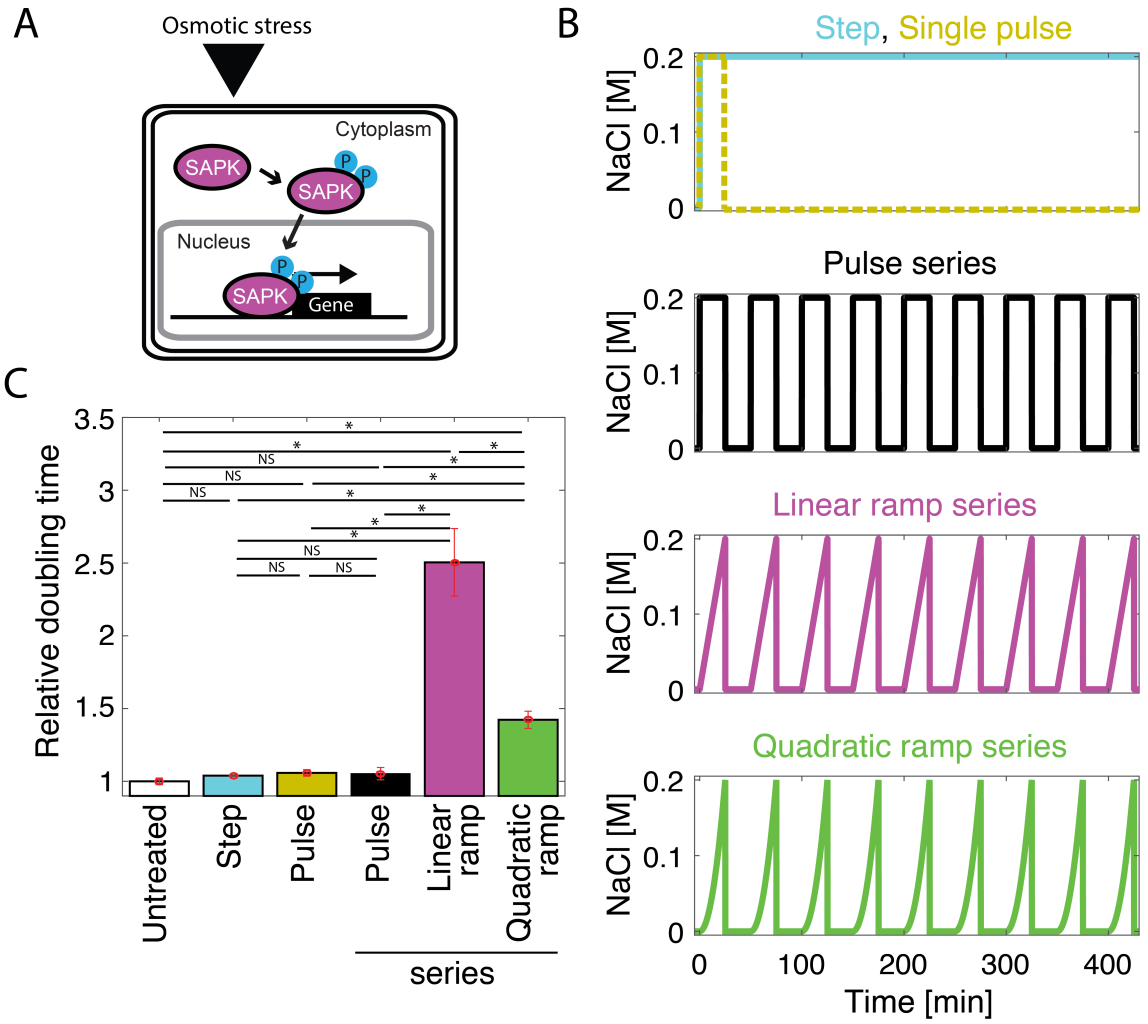

Fig. 1. Growth phenotype upon linear increase of osmotic stress. **(A)** Schematic of osmolyte induction of the Stress Activated Protein Kinase (SAPK) signaling pathway and subsequent gene transcription activation as a model to investigate phenotypic changes upon different environments. **(B)** Temporal extracellular NaCl osmolyte profiles with the same final concentration of 0.2M NaCl (Top to bottom). Single step (cyan line), 25 minute pulse (yellow line), or repetitive series with 25 minutes exposure to the osmolyte as a pulse (black line), a linear ramp (magenta line), and a quadratic ramp (green line), each separated by no osmolyte for 25 minutes. **(C)** Cell growth phenotype as quantified by the relative cell population doubling time relative to untreated cells after exposure of the cells to temporal extracellular NaCl

osmolyte profiles depicted in (B). Cell optical density was measured every 50 minutes. Bars and errors are means and standard deviation from three biological replicates. * one-sided t-test p-values < 0.05. NS: not significant.

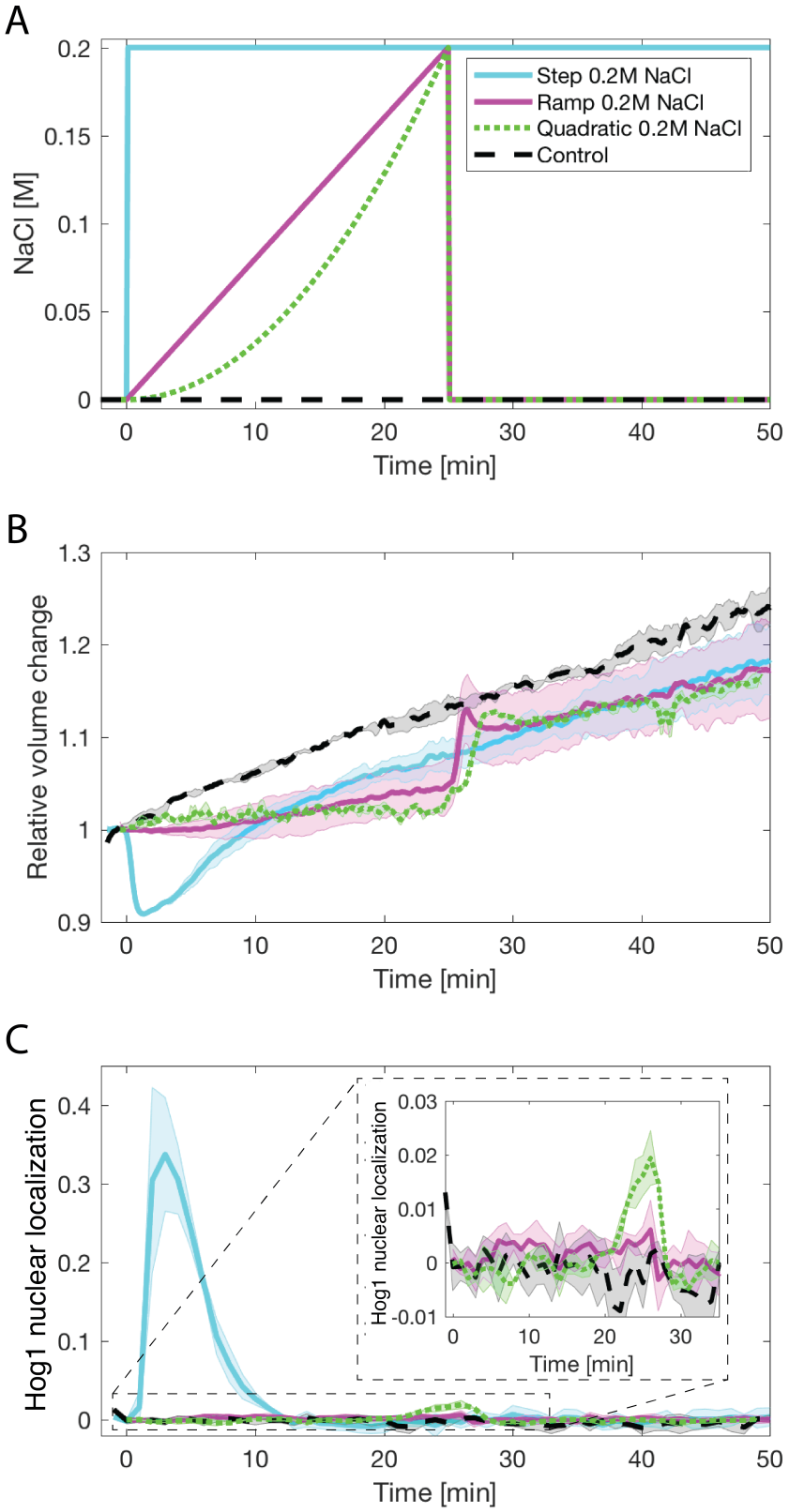
A
NaCl [M]
Time [min]
Step 0.2M NaCl
Ramp 0.2M NaCl
Quadratic 0.2M NaCl
Control
B
Relative volume change
Time [min]
C
Hog1 nuclear localization
Time [min]
Hog1 nuclear localization
Time [min]

Fig. 2. Cells detect extracellular stress but do not active Hog1 signaling in single cells. (A) Osmolyte profiles with a final concentration of 0.2M NaCl are applied to single cells in a microfluidic chamber as a step (cyan line), a linear ramp (magenta line), or a quadratic ramp for 25 minutes (green dashed line). As a control, cells are exposed to media alone (black dashed line). **(B)** Cell volume change relative to t = 0 minutes as a function of time for single cells that are exposed to the same conditions as in (A). **(C)** Hog1 nuclear localization relative to t = 0 minutes as a function of time for single cells that are exposed to the same conditions as in (A). The insert is the enlarged Hog1 nuclear localization. (B, C) Thick lines are the mean and shaded areas are the standard deviation from 3-5 biological replicates. A total of 404 individual cells were analyzed.

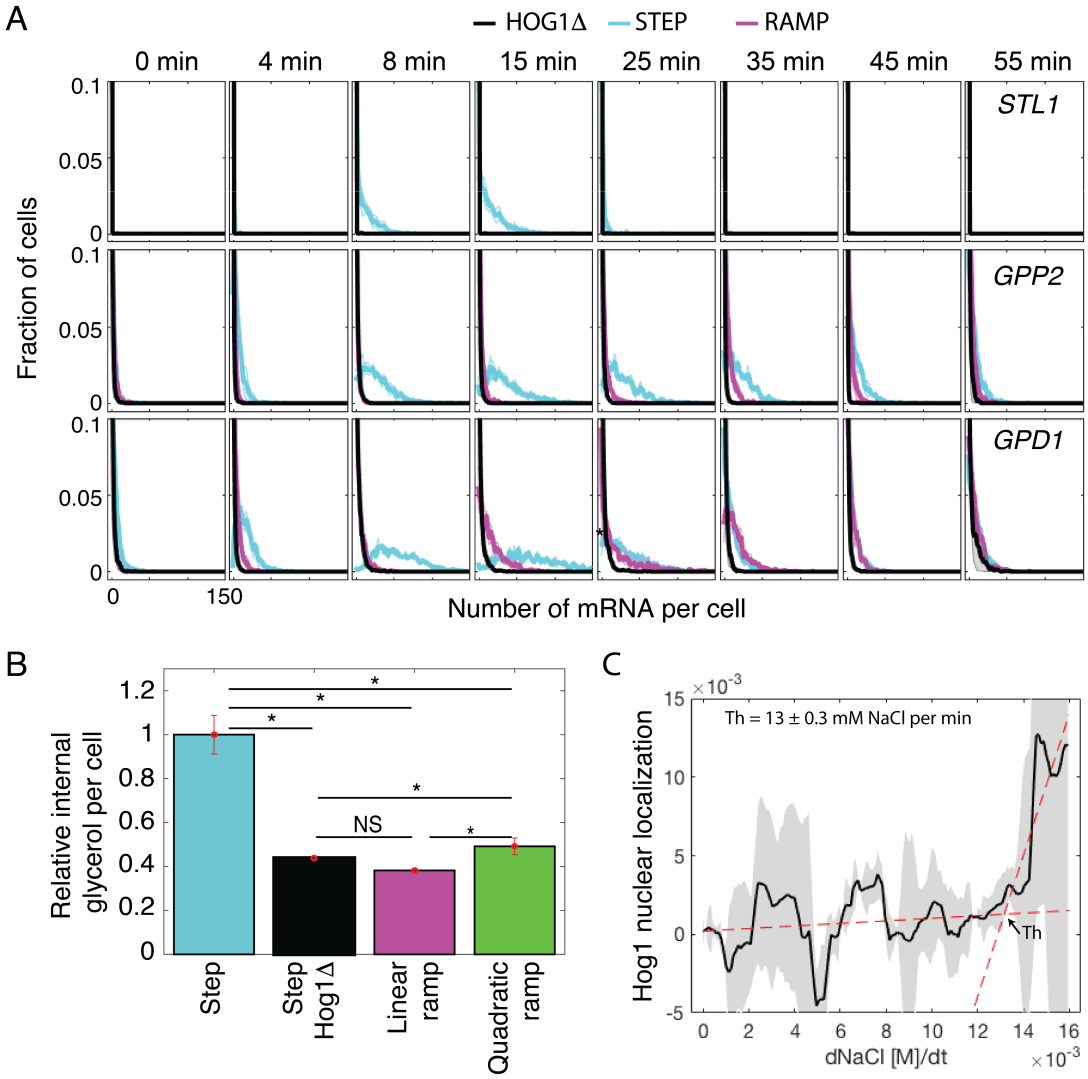

Fig. 3. A threshold rate of osmolyte change is required to activate Hog1 signaling, mRNA transcription and glycerol production. (A) Fraction of cells transcribing *STL1*, *GPP2*, and *GPD1* mRNA as a function of time in single cells measured with single molecule RNA-FISH. Cells were exposed to 0.2M NaCl as a step in WT (cyan line) and Hog1Δ cells (black line), or to a linear ramp for 25 minutes in WT cells (magenta line). Solid lines are the mean and shaded areas are the standard deviation from two biological replicates experiments. A total of more than 82,500 single cells were measured. **(B)** Relative internal glycerol production per cell after 25 minutes exposure of cells to 0.2M NaCl as a step in WT (cyan) and Hog1Δ cells (black), or as a linear (magenta) and a quadratic ramp (green) for 25 minutes in WT cells. Bar plots are means and standard deviations from three biological replicates experiments. *one-sided t-test p-values <

0.05. NS: not significant. **(C)** The threshold rate of external osmolyte change was determined within a single experiment by plotting the Hog1 nuclear localization for cells that are exposed to a quadratic ramp for 25 minutes (Figure 2C, green dashed line) as a function of the first derivative of the quadratic ramp profile (dNaCl/dt) (Figure 2 A, green dashed line). The black line are the mean and the grey area are the standard deviation from two biological replicas. Red dashed lines are piecewise linear fits to the biological replicas and their intercept is defined as the threshold rate (Th). This threshold was validated through ramp experiments below that threshold.

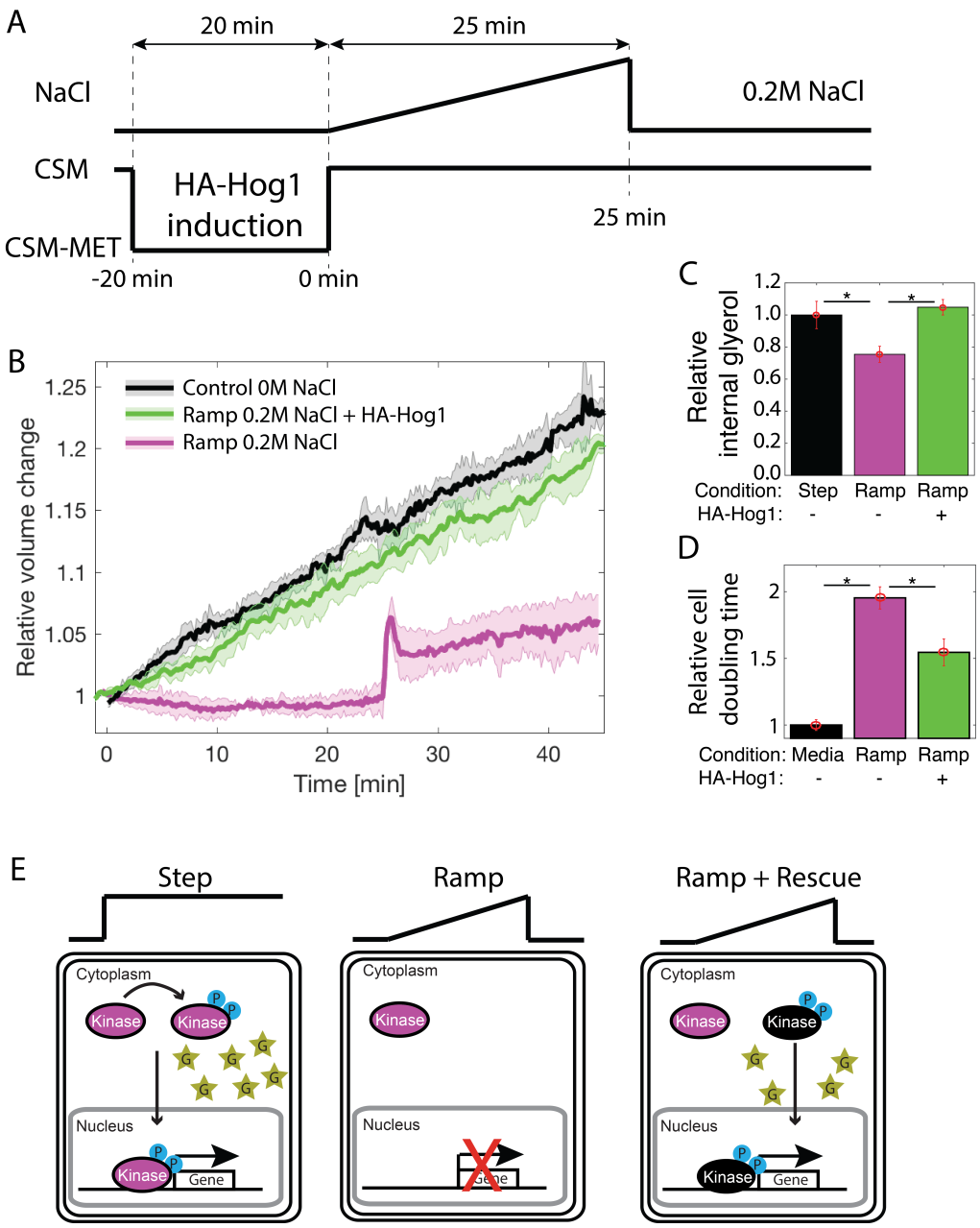

Fig. 4. Transient hyperactive Hog1 rescues phenotypes. (A) Cells were transformed with a plasmid expressing a hyperactive Hog1 kinase (HA-Hog1) that is transiently induced upon removal of methionine from the media. Media (CSM) is switched to media lacking methionine (CSM-MET) 20 minutes before cells are exposed to osmotic stress. After 20 minutes, cells were exposed to CSM with a linear ramp of 8mM NaCl per minute for 25 minutes and then switched back to CSM. **(B)** Relative volume change was measured in single cells starting at t = 0 minutes for cells exposed to media control (black line), to a linear ramp of 8mM NaCl per minute

(magenta line) and to a linear ramp of 8mM NaCl per minute after these cells have been transiently induced for 20 minutes with HA-Hog1 (green line). The solid lines are the mean and the shaded area are the standard deviation from 2-5 biological replicates. A total of 285 cells are measured. **(C)** Relative internal glycerol was measured after 25 minutes for cells that were exposed to a step of 0.2M NaCl (black) or to a ramp to 0.2M NaCl of 25 minutes with (green) and without HA-Hog1 (magenta) induction. **(D)** Relative cell population doubling time measured over 7 hours for cells that are exposed to media (black) or to a repetitive series of ramps to 0.2M NaCl of 25 minutes followed by media without osmolyte for the same time (-HA-Hog1, magenta). Prior to the same type of experiment, cells were transiently induced for 20 minutes with hyperactive Hog1 kinase (+ HA-Hog1, green). Bars are the mean and the errors are the standard deviation from three biological replicates. * one-sided t-test p-values < 0.05 for panel C and D. **(E)** Our working model is that cells that are protected against a NaCl step (left). By contrast, cells can detect a linear NaCl gradient but fail to active Hog1 (purple) via Hog1 phosphorylation and subsequent nuclear translocation, which fails to activate target gene and internal glycerol production, resulting in reduced glycerol (yellow stars) accumulation and slow growth (middle). This phenotype can be rescued through a transient activation of a hyperactive (black) Hog1 (right).