1 **Experimental evolution reveals a general role for the**

2 **methyltransferase Hmt1 in noise buffering**

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23

1 **ABSTRACT**

2 Cell-to-cell heterogeneity within an isogenic population has been observed in

3 prokaryotic and eukaryotic cells. Such heterogeneity often manifests at the

4 level of individual protein abundance and may have evolutionary benefits,

5 especially for organisms in fluctuating environments. Although general

6 features and the origins of cellular noise have been revealed, details of the

7 molecular pathways underlying noise regulation remain elusive. Here, we

8 used experimental evolution of *Saccharomyces cerevisiae* to select for

9 mutations that increase reporter protein noise. By combining bulk segregant

10 analysis and CRISPR/Cas9-based reconstitution, we identified the

11 methyltransferase Hmt1 as a general regulator of noise buffering. Hmt1

12 methylation activity is critical for the evolved phenotype, and noise buffering is

13 primarily achieved via two Hmt1 methylation targets. Hmt1 functions as an

14 environmental sensor to adjust noise levels in response to environmental

15 cues. Moreover, Hmt1-mediated noise buffering is conserved in an

16 evolutionarily distant yeast species, suggesting broad significance of noise

17 regulation.

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1    **Author Summary**

2    Cell-to-cell heterogeneity within an isogenic population has been observed in

3    prokaryotic and eukaryotic cells. Such heterogeneity often manifests at the

4    level of individual protein abundance and may have evolutionary benefits,

5    especially for organisms in fluctuating environments. Here, we used

6    experimental evolution of *Saccharomyces cerevisiae* to select for mutations

7    that increase reporter protein noise and identified the methyltransferase Hmt1

8    as a general regulator of noise buffering. Hmt1 is a central hub protein that is

9    involved in multiple basic cellular pathways, including chromatin

10   remodeling/transcription, translation, ribosome biogenesis, and

11   post-transcriptional regulation. Our results show that Hmt1 constrains the

12   noise level of multiple cellular pathways under normal conditions, so the

13   physiology of individual cells in a population will not deviate too much from

14   optimal peak fitness. However, when cells encounter environmental stresses,

15   HMT1 is quickly down-regulated and expression noise is enhanced to increase

16   the likelihood of population survival. Moreover, the noise buffering function of

17   Hmt1 is conserved in *Schizosaccharomyces pombe* that diverged from the

18   common ancestor of *Saccharomyces cerevisiae* more than 400 million years

19   ago. Since the Hmt1 network is conserved from yeast cells to human, it is quite

20   possible that Hmt1-mediated noise buffering also operates in multicellular

21   organisms.

## 1 Introduction

2 Genetically identical cells grown under homogeneous conditions can still

3 exhibit heterogeneous phenotypes. This heterogeneity is ubiquitous and

4 manifests at different levels, from individual protein concentrations [1] to cell

5 physiology [2, 3]. Although phenotypic heterogeneity only exists transiently, it

6 can lead to deterministic outcomes. In multicellular organisms, a stochastic

7 difference in the initial cell state can result in different cell fates during

8 development [4, 5]. Moreover, stochastic variation in gene expression has

9 been shown to determine the outcome of inherited detrimental mutations [6, 7],

10 representing a possible cause for the incomplete penetrance observed in

11 many human diseases. In microbial cells, levels of pre-existing heterogeneity

12 can influence population fitness upon exposure to unpredictable environmental

13 change [8, 9]. This "bet-hedging strategy" is commonly used by

14 microorganisms to ensure population survival without the fitness cost of

15 developing complex regulatory networks that respond to fluctuating

16 environments [10].

17 At the gene expression level, pre-existing cell-to-cell heterogeneity (or

18 "cellular noise") mainly originates from the stochasticity inherent to molecular

19 processes (such as transcription factor binding to target sequences) and

20 fluctuating levels or activities of factors critical to those processes (such as

21 RNA polymerase II or ribosomes) [1, 11]. Genome-wide studies have shown

22 that low-abundance proteins often present higher expression noise, which is

23 consistent with the greater variability of infrequent events [12, 13].

24 However, some studies have revealed certain pathway-specific patterns

25 in noise levels. For example, housekeeping genes tend to have lower noise,

26 whereas environment-responsive genes are often noisier [14, 15], perhaps

4

because fluctuations in housekeeping genes may compromise essential cellular functions and noisy environment-responsive genes can exert a bet-hedging function. The observed patterns in these two types of genes indicate that selection operates on protein noise levels or levels have been adjusted according to potential costs and benefits over the course of evolution. Moreover, a study comparing young and old mice showed that heart cells isolated from old mice exhibit higher gene expression noise than those isolated from young mice [16], suggesting that noise levels are tightly controlled in young healthy cells but the control systems deteriorate with age.

How do cells adjust protein noise? Several general features have been associated with expression noise including network topology, cellular compartmentalization, molecular chaperone abundance, nucleosome occupancy, and promoter architecture [7, 17-19]. Genetic studies have also identified mutations that alter local or general noise levels [5, 11, 20, 21]. Nonetheless, how cells respond to growth conditions and integrate different pathways to fine-tune protein noise remains insufficiently characterized.

To understand how protein noise is regulated, we used experimental evolution of *Saccharomyces cerevisiae* to search for mutations that increased the protein noise of different reporter genes. After 35 cycles of selection, two of the evolved lines (*TDH2-GFP-* and *TYS1-GFP*-carrying lines) exhibited increased noise levels without a concomitant reduction in protein abundance. We show that increased protein noise in the evolved line carrying the *TDH2* reporter gene is not specific to the *TDH2*-related pathway, suggesting that the evolved mutations have a general effect on protein noise regulation. We identified the methyltransferase Hmt1 as the major contributor of the evolved phenotype. Further experiments revealed that noise regulation is mediated by

1    methylation of multiple downstream targets of Hmt1 and that *HMT1* expression

2    is often attenuated under stress conditions. Our results suggest that Hmt1

3    functions as a master coordinator of bet-hedging strategies in response to

4    environmental stress.

5

6    **Results**

7    **Experimental evolution of increased protein noise in budding yeast**

8    A previous study showed that alternating selection between highest- and

9    lowest-expression subpopulations could efficiently enrich promoter variants for

10   high transcriptional noise in bacterial cells [22]. We hypothesized that a similar

11   selection strategy might allow us to "evolve" yeast cells to increase the protein

12   noise of reporter genes (Fig. 1A). Eight genes (*ADK1*, *APA1*, *PCM1*, *RPL4B*,

13   *SAM4*, *TDH2*, *TPD3*, and *TYS1*) selected from distinct cellular pathways were

14   fused with GFP to generate our reporters (see Materials and Methods).

15   Evolving lines carrying individual reporter genes were subjected to alternating

16   selection between the top 5% and bottom 5% of total populations in terms of

17   their GFP intensity. We also treated cells with a mutagen (2.8% ethyl

18   methanesulfonate, EMS) before each selection cycle to increase the genetic

19   diversity of evolving populations. After 35 cycles of selection, half of the

20   evolved lines (including the strains carrying *RPL4B*, *TDH2*, *TPD3*, or *TYS1*

21   reporter genes) exhibited significantly increased reporter noise (Fig. 1B).

22          Among them, the Tdh2-GFP- and Tys1-GFP-carrying lines also presented

23   increased mean protein intensity, ruling out the possibility that the increased

24   noise was due to reduced mean protein signal intensity. These results indicate

25   that our selection regime effectively increased protein noise upon experimental

1  evolution. In order to dissect the genetic basis of noise regulation, we selected

2  the Tdh2-GFP-carrying line for further analysis since it exhibited the greatest

3  increase in noise without a concomitant decrease in mean signal intensity.

4

5  **Isogenic cells with high and low Tdh2 levels have fitness advantages**

6  **under different conditions**

7  Bet-hedging is a commonly adopted survival strategy among microorganisms

8  for spreading the risk of encountering hostile environments [9, 23]. Tdh2

9  protein is a glyceraldehyde-3-phosphate dehydrogenase involved in

10  glycolysis/gluconeogenesis and it has been shown to help cells resist oxidative

11  stress during the stationary phase [24]. We tested if cells with high or low

12  Tdh2-GFP levels represent different physiological states and if they exhibit

13  fitness advantages under different conditions. To do this, we isolated individual

14  stationary phase cells presenting different levels of Tdh2-GFP using a cell

15  sorter and examined their phenotypes.

16  Consistent with a previous observation [24], upon $H_2O_2$ treatment, cells

17  with high Tdh2-GFP levels had higher survival rates than those with low

18  Tdh2-GFP (Fig. 2A). Interestingly, when we provided fresh nutrients, the cells

19  with high levels of Tdh2-GFP tended to re-enter the cell cycle more quickly

20  than those with low Tdh2-GFP levels, despite cells with different levels of

21  Tdh2-GFP exhibiting no difference in survival rates under this condition (Fig.

22  2B). This variation in cell cycle re-entry is reminiscent of the divergent

23  germination times observed in same populations of plants or fungi, which has

24  been suggested to be a risk-spreading strategy to enhance long-term survival

25  [25-27]. Divergent cell cycle re-entry times can prevent an entire cell

1 population from going extinct upon occurrence of an unpredicted

2 environmental catastrophe. To test this hypothesis, we collected cells

3 displaying either high or low Tdh2-GFP and challenged the cells with heat

4 stress either before or after the cells had been re-fed with fresh nutrients. We

5 found that cells with low Tdh2-GFP had a survival rate 3-fold greater than that

6 of cells with high Tdh2-GFP upon encountering heat stress after nutrient

7 refreshment. Survival rates were similar and independent of Tdh2-GFP levels

8 for cells either non-stressed or stressed before nutrient refreshment and (Fig.

9 2C).

10     We also found that high and low levels of Tdh2-GFP represent transient

11 states, but not genetic modifications, of the cells. When we propagated cells

12 sorted into high and low Tdh2-GFP populations, Tdh2-GFP intensities of both

13 populations reverted to a level similar to that of the initial unsorted population

14 after a few generations (Fig. S1). Together, our results demonstrate that

15 having cells with different levels of Tdh2 in an isogenic population is

16 advantageous in different environments, revealing the risk-spreading benefit of

17 expression noise.

18

19 **Increased noise is not limited to the *TDH2*-related pathway in the**

20 **Tdh2-GFP evolved line**

21 Before characterizing the detailed phenotypes of the evolved

22 Tdh2-GFP-carrying line, we examined the cell populations and found that the

23 signal for increased noise was not bimodal (Fig. S2A). We further confirmed

24 that Tdh2-GFP retained its full length and subcellular localization after

25 evolution (Fig. S2B and S2C), and that there were no mutations in its promoter

1     or coding regions. These data indicate that the increased noise in the evolved

2     line is not caused by mutations in the *TDH2* locus.

3       If the evolved mutations in the Tdh2-GFP-carrying line occurred within

4     sequences pertaining to general noise regulators, these mutations may affect

5     both the *TDH2*-related pathway and other pathways. We selected four

6     pertinent genes—*TDH3*, *PGK1*, *ADK1*, and *GLY1*—to investigate the effect of

7     the evolved mutations. *TDH3* is a paralog of *TDH2* that encodes an enzyme for

8     glycolysis/gluconeogenesis. *PGK1* encodes another key enzyme involved in

9     glycolysis/gluconeogenesis. Both *TDH3* and *PGK1* are likely co-regulated with

10     *TDH2* since all three respective proteins operate in the same metabolic

11     pathway [28]. *ADK1* encodes an adenylate kinase required for purine

12     metabolism [29], and *GLY1* encodes a threonine aldolase involved in glycine

13     biosynthesis [30], neither of which is related to the *TDH2* pathway.

14     Nonetheless, all four genes exhibited increased protein noise in the evolved

15     line (Fig. 3A), suggesting that the effect of the evolved mutations is not

16     pathway-specific.

17       We also subjected a *TDH2* promoter-driven GFP construct to the same

18     analysis and observed increased noise in the respective evolved line (Fig. 3B),

19     raising the possibility that increased noise in the evolved cell lines may be

20     attributable, at least in part, to promoter regulation or mRNA turnover.

21

22     **An Hmt1 methyltransferase mutant significantly contributes to the**

23     **evolved increase in noise**

24     To understand the genetic basis of the increased noise we observed in the

25     evolved Tdh2-GFP-carrying line, both ancestral and evolved lines were

1    subjected to whole genome sequencing. A total of 1022 mutations (including

2    494 non-synonymous, 256 synonymous, and 271 intergenic mutations) were

3    identified in the evolved genome (Table S1). This high number of mutations is

4    most likely due to the mutagen treatment we applied during the cycles of

5    selection.

6         Next, we used bulk segregant analysis to refine the list of candidate

7    mutations. To do this, we crossed the evolved line to the ancestral line and

8    analyzed their F1 haploid progeny. We measured the noise level of Tdh2-GFP

9    in 360 segregants and established an "evolved-like" pool (comprising 16

10    segregants) and an "ancestral-like" pool (20 segregants; see Fig. S3 and

11    Materials and Methods). Both of these segregant pools were then subjected to

12    whole genome sequencing. Based on our computational simulation, we

13    applied two criteria to select candidate mutations from these segregant pools:

14    1) the mutation frequency in the "evolved-like" pool had to be >70%; and 2) the

15    difference in mutation frequencies between the "evolved-like" and

16    "ancestral-like" pools was >38% (see Materials and Methods). Twenty

17    non-synonymous mutations met these two assumptions and were subjected to

18    reconstitution experiments (Table S2).

19         We first introduced the candidate mutations into the ancestral line using

20    the CRISPR/Cas9 system and then examined the noise level of Tdh2-GFP.

21    Among the tested reconstitution lines, we found that a mutation (G70D) in the

22    methyltransferase-encoding gene, *HMT1*, resulted in a significant increase of

23    noise, close to the level exhibited by the evolved line (Fig. 4A). Similarly, we

24    observed increased noise in the *TDH2* promoter-driven GFP construct (Fig.

25    4B). When we rescued the mutation in the evolved line by reverting to the

26    wild-type sequence, Tdh2-GFP noise was reduced to a level similar to the

1    ancestral line (Fig. 4A). These data indicate that *hmt1-G70D* is a primary

2    mutation contributing to the evolved increase in noise.

3        Already in this study we have shown that proteins in both *TDH2*-related

4    and unrelated-pathways exhibited increased noise in the evolved line (Fig.

5    3A). In the *hmt1-G70D* reconstitution line, we observed similar noise increases

6    in the *TDH3*, *ADK1*, and *GLY1* reporter genes (Fig. 4C), indicating that the

7    increased noise observed in the evolved line is mainly due to the *hmt1-G70D*

8    mutation.

9

10   **The noise buffering effect of Hmt1 is mediated through multiple**

11   **methylation targets**

12   Hmt1 is a methyltransferase that methylates arginine residues in its

13   substrates. The mutated G70D residue is located within a conserved

14   methyltransferase motif (Fig. S4A), and mutations in this motif cause loss of

15   methyltransferase activity in *E. coli* and yeast [31, 32]. The *hmt1-G70D* mutant

16   also exhibited a noise increase similar to levels exhibited by *HMT1*-deletion

17   cells (Fig. S4B). Moreover, Western blot analysis using anti-methylated

18   arginine antibodies confirmed that *hmt1-G70D* mutant cells lack Hmt1

19   methyltransferase activity (Fig. S4C). These results suggest that the noise

20   regulating function of Hmt1 is mediated through its methyltransferase activity.

21       In vivo and in vitro studies have identified several Hmt1 substrates [33-35].

22   Moreover, Hmt1-mediated methylation has been shown to enhance the

23   function of its substrates in multiple pathways, including chromatin remodeling

24   and transcription [36-38], translation and ribosome biogenesis [39-42], and

25   post-transcriptional regulation [43-46]. We selected the representative proteins

1    Npl3, Rps2, Sbp1, and Snf2 from among these pathways and generated

2    corresponding deletion or hypomorphic mutants (in cases where mutant

3    haploids died or had severe growth defects) and investigated noise levels

4    among these mutants.

5        Of all these tested mutants, only the *rps2* and *snf2* mutants presented

6    significantly increased noise (Fig. 5A), with the other mutants showing either

7    no change or reduced noise (Fig. 5B). Rps2 is a component of the small

8    ribosomal subunit, suggesting involvement of translational regulation in noise

9    control. Snf2 is the catalytic subunit of the SWI/SNF chromatin remodeler and

10    its function depends on two other SWI/SNF components, Snf5 and Snf6 [47,

11    48]. We further assessed *snf5* and *snf6* mutants and found that they also

12    presented increased noise (Fig. 5A and 5C), confirming the general role

13    played by the SWI/SNF chromatin remodeler in noise regulation.

14        To test whether *hmt1-G70D* mutation affects the activity of SWI/SNF

15    complexes, we performed a functional assay of the SWI/SNF chromatin

16    remodeler. It has been shown previously that transcriptional activation of the

17    *CHA1* gene is attenuated when cells have defective SWI/SNF complexes [49].

18    We monitored mRNA levels of *CHA1* immediately after shifting cells to a

19    *CHA1*-inducing medium. Expression of *CHA1* was indeed reduced in

20    *hmt1-G70D* mutant cells and in *snf5* mutants (Fig. 5D), indicating that the

21    function of SWI/SNF complexes is compromised in *hmt1-G70D* mutant cells.

22        We then examined the promoter regions of *TDH2* and *GLY1* (unrelated to

23    *TDH2*) by histone chromatin immunoprecipitation (ChIP) combined with

24    quantitative PCR to understand how Hmt1 influences gene expression. We

25    observed increased nucleosome occupancy in *hmt1-G70D* mutant cells (Fig.

26    5E), providing evidence for the supposition that noise regulation partially

1  operates via SWI/SNF-mediated nucleosome remodeling [18].

2

**Hmt1 functions as a mediator in responses to environmental stresses**

Genome-wide studies have indicated that *HMT1* expression frequently fluctuates under different growth conditions [50, 51], suggesting that cells may employ an environmental sensor to enhance phenotypic heterogeneity upon encountering stress. We measured the mRNA levels of *HMT1* under various stress conditions—including heat, oxidative stress, high osmolarity, and glucose starvation—and found that they were significantly reduced under all of these conditions (Fig. 6A). Consistent with the concept of an environmental sensor, the noise level of Tdh2-GFP was also increased under stress conditions (Fig. 6B and S5). Together, these data indicate that Hmt1 can serve as a mediator to control levels of phenotypic heterogeneity in response to environmental stimuli.

Our data show that bet-hedging within a population could be represented by the noisy expression of Tdh2-GFP (Fig. 2). If increased noise can help populations survive stressful environments, we anticipated that cells with low Hmt1 activity under stress would present an enhanced survival rate relative to those with normal Hmt1 activity under stress. Indeed, when we treated wild-type and *hmt1-G70D* mutant cells with $H_2O_2$, the mutant population exhibited better viability (Fig. 6C).

**Hmt1-mediated noise buffering is conserved in *Schizosaccharomyces pombe***

13

1    Our results suggest that the methyltransferase Hmt1 may function as a general

2    noise regulator that constrains physiological noise in normal conditions but

3    facilitates heterogeneity under stress. This buffering mechanism is likely to

4    increase long-term population survival. Methyltransferase is a conserved

5    enzyme that exists in all eukaryotic kingdoms. We examined whether its

6    buffering function is also conserved in a phylogenetically distinct species, *S.*

7    *pombe*, which diverged from the common ancestor of *S. cerevisiae* at least

8    400 million years ago [52]. We generated YFP fusion protein constructs of *tdh1*

9    and *gpd3* (the *S. pombe* orthologs of *TDH2*) and examined their noise levels in

10   the wild-type and *rmt1* (the *S. pombe* ortholog of *HMT1*) deletion backgrounds.

11   Similar to our findings for *S. cerevisiae* evolved lines, noise levels of Tdh1-YFP

12   and Gpd3-YFP were significantly increased in *rmt1* mutant cells (Fig. 6D).

13   Accordingly, the Hmt1-mediated noise buffering system probably represents

14   an   important   survival   strategy   that   is   conserved   across   diverse

15   microorganisms.

16

17   **Discussion**

18   Microorganisms constantly face changing environments. Despite being

19   equipped with complex stress-adaption systems, unpredicted acute stress

20   remains a challenge for cells. Cell populations harboring heterogeneous

21   physiological states enhance their likelihood of surviving environmental

22   fluctuations [53, 54]. Many examples of bet-hedging have been reported

23   previously, indicating that this is a common survival strategy among microbes

24   [5, 55]. However, it is not known if cells exhibit another layer of regulation that

25   allows them to adjust their levels of noise. Our current study provides evidence

14

1    that Hmt1 can function as a core regulator to constrain or facilitate phenotypic

2    heterogeneity in response to environmental stimuli.

3        The evolutionary advantage of an environment-sensing noise regulator is

4    readily conceivable. Although stochastic noise is inevitable among individuals

5    within a population, excessive deviation from "normal" levels may result in a

6    significant reduction of fitness under normal conditions. By regulating multiple

7    pathways, Hmt1 allows cells to constrain noise levels. However, when a

8    population is exposed to mild stresses, Hmt1 expression is immediately

9    down-regulated and the noise buffering system is curtailed. The resulting

10   enhanced heterogeneity means that the population increases its likelihood of

11   survival, especially if the stress is prolonged or escalates (Fig. 7).

12       Hmt1 is a type-I arginine methyltransferase that catalyzes

13   mono-methylation and asymmetric di-methylation in budding yeast.

14   Hmt1-mediated methylation has been shown to influence various cellular

15   pathways including transcription, post-transcription and translation [36-38, 40,

16   42-46]. Moreover, Hmt1 is a highly interactive protein, ranking among the top

17   1.5% of the entire budding yeast proteome in terms of the interaction number

18   [56], suggesting it has a central role in coordinating a complex network.

19   Previous studies have shown that another network hub, Hsp90, can regulate

20   general cellular noise [17, 57]. Consistent with predictions from network

21   analyses and results from a genome-wide screen, hub genes have a strong

22   impact on buffering non-genetic and genetic variation [58-60]. More

23   interestingly, both Hsp90 and Hmt1 can work as environmental sensors to

24   adjust noise levels in response to environmental cues, acting as direct links

25   between noise regulation and adaptive benefits.

26       Our results show that at least two downstream pathways, i.e., chromatin

1  remodeling and the translational machinery, are involved in Hmt1-mediated

2  noise buffering. The effect of the SWI/SNF chromatin-remodeling complex on

3  gene noise has been reported previously [11]. Here, we have identified Hmt1

4  as an upstream coordinator of noise regulation and establish the novel role of

5  protein methylation in this process. The detailed mechanism underlying how

6  the ribosomal component Rps2 regulates noise is less clear. Rps2 plays a

7  crucial role in controlling translational accuracy and its efficiency can be further

8  modulated by post-translational modifications [61, 62]. It is possible that

9  Rps2-mediated noise regulation occurs at the protein translation level. More

10  experiments are required to address this issue.

11      The regulatory functions of Hmt1 are generally well conserved in human

12  cells, but they are executed by six orthologs [33, 63]. Human orthologs also

13  exist for many Hmt1 substrates, which interact respectively with different

14  human type I enzymes [34, 64-66]. For example, the human Hmt1 ortholog

15  PRMT4 interacts with the Snf2 ortholog BRG1 to facilitate the ATPase activity

16  of the entire human SWI/SNF complex [66]. It is likely that the interactions

17  between Hmt1 and its substrates represent an ancient regulatory network. In

18  support of this supposition, we observed increased noise in *S. pombe* and *S.*

19  *cerevisiae hmt1* mutants, suggesting that Hmt1-mediated buffering evolved

20  hundreds of millions of years ago before the ancestors of these two species

21  diverged. Hmt1-mediated noise buffering may also help populations of

22  unicellular *S. pombe* to survive in stressful environments. However, with

23  regard to multicellular organisms, methylation-regulated noise buffering may

24  influence their developmental plasticity, and this interesting topic awaits further

25  study.

26

1 **Materials and Methods**

2 **Yeast strains and genetic procedures**

3 All *Saccharomyces cerevisiae* strains used in this study were derived from the

4 W303 strain (*leu2-3, 112 trp1-1 can1-100 ura3-1 ade2-1 his3-11, 15*), and the

5 *Schizosaccharomyces pombe* strains were derived from the 972 h- strain.

6 Unless otherwise indicated, gene deletion or insertion was based on

7 homologous recombination. Yeast cells were transformed using the Lithium

8 acetate method [67] or electroporation under 1800 V/ 200 $\Omega$/ 25 $\mu$F (BTX

9 Gemini SC$^2$, Fisher Scientific, Pittsburgh, PA) [68].

10 All fluorescent fusion proteins were constructed by chromosomal in-frame

11 insertion of the fluorescent protein tag at the 3' end of the coding region of

12 target genes. The GFP tags were directly amplified from the genomic DNA of

13 corresponding strains in the yeast GFP collection [69]. The BFP tag was

14 amplified from the plasmid pFA6a-link-yomTagBFP2-Kan [70]. Prior to

15 constructing *ADK1-GFP* and *TDH2* promoter-driven *GFP* in ancestral and

16 evolved *TDH2-GFP*-carrying lines, the *GFP* of *TDH2-GFP* in these strains was

17 removed and replaced with a stop codon and the *TDH2* 3' untranscribed

18 region (UTR). For *TDH2* promoter-driven *GFP*, 1000 base pairs (bp) upstream

19 of the *TDH2* coding region was fused with *GFP*, and the fused fragment was

20 inserted between positions 204886 and 204887 of chromosome I [71]. For

21 YFP-tagging in *S. pombe*, the coding region of yVenus [72] without the start

22 codon was fused with hphMX6 by two-fragment PCR. To delete *S. cerevisiae*

23 genes, KanMX4-containing DNA fragments for homologous recombination

24 were amplified from the genomic DNA of corresponding strains in the yeast

25 deletion collection [73]. To construct a hypomorphic mutant of *RPS2*, we

17

1  directly amplified the *rps2*-DAmP allele from the yeast DAmP diploid collection

2  [74] and used it to replace the native gene. To delete *rmt1* in *S. pombe*, a

3  KanMX6-containing DNA fragment with homologous flanking regions was

4  generated and used to replace the whole gene.

5  All SNP mutant reconstitution strains were constructed using the

6  CRISPR/Cas9 system [75, 76]. Briefly, host cells were transformed with the

7  Cas9 expression plasmid and then the transformant was introduced with a

8  DNA fragment encoding gRNA, a linearized vector, and mutation-containing

9  donor DNA fragments. After transformation, single colonies were streaked out

10  on YPD (1% Yeast extract, 2% Peptone and 2% Dextrose) plates to purify the

11  CRISPR transformants. Genomic DNA of the resulting single colonies was

12  isolated and examined initially by allele-specific PCR [77] and then by Sanger

13  sequencing. The plasmids for Cas9 and gRNA expression are listed in Table

14  S3.

15

16  **Experimental evolution**

17  For the evolution experiment, eight genes (*ADK1*, *APA1*, *PCM1*, *RPL4B*,

18  *SAM4*, *TDH2*, *TPD3*, and *TYS1*) were selected and fused with GFP to

19  generate evolving strains each carrying an individual reporter gene. These

20  reporter genes were chosen based on the following criteria: 1) each gene

21  belongs to a distinct cellular pathway; 2) the expression level of GFP fusion

22  proteins is constant during the cell cycle and is sufficiently high to be

23  detectable by flow cytometry; and 3) the reporter proteins are evenly localized

24  in the nucleus or cytosol to avoid misinterpretation due to dynamic organelles.

25  Due to the complexity of our selection regime, only one evolving line for

1    each reporter gene was established. In each selection cycle, $1 \times 10^6$ cells were

2    treated with 2.8% EMS (see the "EMS mutagenesis" section below for details)

3    to increase the genetic diversity of the cell population. This mutagenic

4    treatment is crucial, as we initially ran a pilot experiment using a similar

5    selection regime but lacking the mutagen treatment and did not observe any

6    obvious increase in noise after 70 cycles of selection. However, our EMS

7    treatments also significantly increased the number of mitochondria-defective

8    cells, which are known to increase population heterogeneity. To constrain the

9    population of mitochondria-defective cells, we grew cells in non-fermentable

10   YPG (1% Yeast extract, 2% Peptone and 2% Glycerol) medium at 28 °C after

11   EMS treatments. After 12 h of growth in YPG, cells were sorted to select 5000

12   cells from the top (or bottom) 5% of the total population in terms of GFP

13   intensity (see the "Fluorescence-activated cell sorting" section below for

14   details). These cells were grown in 3 ml YPG for ~36 h to reach $OD_{600}$ = 1

15   before proceeding to the next cycle of selection. We used alternating selection

16   between the top 5% and bottom 5% of total populations throughout the

17   evolution experiment. The effective population size was estimated to be 1.33 ×

18   $10^5$ cells using the formula $2/N_e = 1/(N_{01} \times g) + 1/(N_{02} \times g)$, in which $N_0$ is the

19   initial population size and $g$ is the number of generations during each growth

20   period [78].

21       After 35 cycles of selection, five individual clones were isolated from each

22   evolved population and their GFP noise levels were measured. The clone with

23   the greatest increase in noise without exhibiting a decrease in the mean

24   intensity of GFP or increased variation in cell size was selected for further

25   genetic analysis.

26

1    **Ethyl methanesulfonate (EMS) mutagenesis**

2    Mutagenesis was performed according to a previously published protocol by

3    which mutation rates can be increased without inducing considerable cell

4    death [79]. Briefly, we washed $1 \times 10^6$ cells with sterile water once, with 100 $\mu$l

5    of phosphate buffer (0.1M $Na_2HPO_4$, pH=7.0) once, and then resuspended

6    them in 90 $\mu$l of phosphate buffer. We added 90 $\mu$l of EMS-containing

7    phosphate buffer to the cell solution, resulting in a final concentration of 2.8%

8    EMS. The solution was maintained under constant shaking at room

9    temperature for 30 min, before stopping the reaction by adding 50 $\mu$l of 25%

10   sodium thiosulfate. After washing with sterile water, the cells were transferred

11   to 10 ml YPG. The survival rate of wild-type cells after EMS treatment ranged

12   from 60% to 80%.

13

14   **Fluorescence-activated cell sorting and flow cytometry**

15   Cells were suspended in filtered phosphate-buffered saline (PBS) (137 mM

16   NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$) and sampled using

17   a BD FACSJazz™ machine (BD Bioscience, Franklin Lakes, NJ) at an event

18   rate of $\leq$ 3000 cells/sec. GFP readouts were acquired with bandpass filters of

19   513/17 nm using laser excitation at 488 nm. For YFP, we used laser excitation

20   at 488 nm with a 542/27 nm filter and for BFP we used a 450/50 nm filter and

21   laser excitation at 405 nm.

22         To eliminate interference from small particles in the fluidic system, we

23   excluded particles with forward scatter (FSC) signals <2570 (as a trigger

24   threshold). We established three hierarchical gates. First, to avoid cell

1   aggregates, we collected cells that had relatively constant signals of trigger

2   pulse width along FSC signals. Second, to eliminate possible cell aggregates,

3   we collected cells that had relatively constant signals of FSC-W along FSC

4   signals. Third, to avoid cell debris and abnormally large cells, we collected

5   cells having FSC and side scatter (SSC) signals both ranking within 5%-95%

6   of the population.

7       For alternating selection in experimental evolution, we applied the trigger

8   threshold and the first and third gates. Based on the distribution of GFP levels,

9   we defined the highest or lowest 5% of the total gated population and collected

10  5000 cells. The collected cells were then grown in 3 ml YPG and used for the

11  next run of selection. For fitness assays, we applied the trigger threshold and

12  all three gates. Based on the distribution of GFP levels, we defined the highest

13  and lowest 5% of the total gated population and collected individual cells. A 1.0

14  single-drop sorting mode was employed. The viability of the collected cells was

15  then measured under different conditions.

16

17  **Noise and signal distribution measurement**

18  We measured the noise of fluorescent protein signals by the Fano factor (a

19  ratio of variance to the mean, %) using more than 5000 early log-phase cells.

20  This measurement is characterized by having less interference from the mean

21  and is more sensitive to variation-driven increased noise [11, 80]. We used the

22  same trigger threshold and the first two hierarchical gates as employed for cell

23  sorting in our noise measurement. For the third gate, we used contour plots of

24  FSC and SSC signals to collect cells constituting 60% of the second-gated

1    population to ensure more homogenous cell size and cell physiology.

2    Fluorescence readouts of the entire population were log-transformed and used

3    to calculate the noise.

4

5    **Fitness assays under different growth conditions**

6    To measure $H_2O_2$ resistance, Tdh2-GFP-carrying cells were grown in YPD for

7    5 days to ensure that the cells had entered stationary phase. More than 100

8    cells from the top 30% or bottom 30% of the gated population in terms of their

9    Tdh2-GFP intensity were sorted and spotted onto plates with or without 4.4

10    mM $H_2O_2$. The survival rates were measured after 5 days of incubation at 28

11    °C.

12    For heat resistance assays, cells from the top 10% or bottom 10% in

13    Tdh2-GFP intensity were sorted into microcentrifuge tubes. The collected cells

14    were divided into two parts. One part was immediately placed in a PCR

15    machine to perform heat ramping from 30 °C to 56 °C for 20 min as a heat

16    stress [81]. The other part was placed in fresh YPD media for 2.5 h and then

17    subjected to the same heat treatment. Cell survival rates were measured as

18    colony-forming units after 3 days of incubation at 28 °C.

19    As a rebudding assay, more than 100 unsorted stationary-phase cells

20    were placed on agarose pads and monitored by time-lapse microscopy at 15

21    min intervals for at least 12 h [82]. Only unbudded cells at the beginning of the

22    recording period were monitored.

23

24    **Western blotting**

25    Log-phase cells were lysed using NaOH lysis [83]. Proteins in the lysates were

22

1     separated with SDS-PAGE and transferred to a PVDF membrane. Mouse

2     anti-GFP antibody (1:4000) (#sc-9996, Santa Cruz Biotechnology, Dallas, TX)

3     was used to detect Tdh2-GFP. Rabbit anti-G6PDH antibody (1:4000) (#A9521,

4     Sigma-Aldrich, St. Louis, MO) was used to detect G6PDH, which served as an

5     internal control. Mouse anti-methylated arginine antibody (mab0002-P,

6     Covalab, Villeurbanne, France) was used to detect proteins with methylated

7     arginine. MultiMab rabbit monoclonal mix antibodies against an asymmetric

8     di-methyl arginine motif (#13522, Cell Signaling Technology, Danvers, MA)

9     were used to detect proteins with asymmetric di-methylated arginine.

10

11     **Microscopy**

12     Microscopy was conducted using a 60x objective lens and an ImageXpress

13     Micro XL system (Molecular Device, Sunnyvale, CA).

14

15     **F1 segregant analysis**

16     The evolved line was mated with the ancestral line, and the resultant diploid

17     cells were induced to sporulate. Sporulated culture was harvested into a

18     microcentrifuge tube and treated with 0.5 mg/ml Zymolyase-100T (Nacalai

19     Tesque Inc., Kyoto, Japan) in 1M sorbitol at 28 °C for 2 h to remove the ascal

20     wall. Cells were then treated with 2% SDS at 28 °C for 10 min to kill

21     unsporulated diploid cells, before washing with sterile water and vortexing

22     vigorously to attach individual spores to the tube wall. We then added 0.01%

23     Triton X-100 solution to the tube, before vigorous sonication to detach spores

24     from the tube wall and to separate spore clusters. The suspension was diluted

25     and spread on YPD plates to isolate F1 haploid segregants.

1    We conducted a total of three runs of noise measurement to identify

2    "evolved-like" and "ancestral-like" F1 segregants. The progenies ranking within

3    the top 20% or bottom 20% of the Tdh2-GFP noise level without changing the

4    mean intensity (i.e., within three standard deviations of the control) were

5    selected for the next run of noise measurement. We started with 360 F1

6    progeny of confirmed ploidy in the first round, and obtained 16 "evolved-like"

7    segregants and 20 "ancestral-like" segregants after finishing the third run.

8    These cells were subjected to whole genome sequencing analysis.

9

10   **Whole genome sequencing analysis**

11   Yeast cells were resuspended in 200 $\mu$l of lysis buffer (2% Triton X-100, 1%

12   SDS, 100 mM NaCl, 10 mM Tris-HCl pH=8.0, and 1 mM $Na_2EDTA$). Glass

13   beads (0.5 mm in diameter) that amounted to the volume of the cell pellet were

14   added, followed by the addition of 200 $\mu$l of PCIA (Phenol:chloroform:isoamyl

15   alcohol=25:24:1, pH=8.0). The mixture was vortexed for 5 min. We then added

16   200 $\mu$l of TE buffer (10 mM Tris·Cl; 1 mM $Na_2EDTA$ pH=8.0) and the mixture

17   was centrifuged for 5 min at maximum speed. Only 350 $\mu$l of the solution in the

18   aqueous layer was extracted and subjected to EtOH precipitation. The pellet

19   was air-dried and then dissolved in 400 $\mu$l of TE buffer containing 75 $\mu$g/ml

20   RNase A. The mixture was then incubated at 37 °C for 5 min. We then added

21   200 $\mu$l of PCIA and we inverted the tubes to mix. EtOH precipitation was then

22   performed at room temperature under constant shaking for 2 h. The pellet was

23   air-dried and then dissolved in 100 $\mu$l of sterile double-distilled water. The ratio

24   of $OD_{260}$ to $OD_{280}$ of the purified DNA ranged from 1.8 to 2.1. Concentrations of

25   genomic DNA were determined using a Qubit™ dsDNA BR assay kit

1    (ThermoFisher Scientific).

2    Equal amounts of genomic DNA from individual "evolved-like" or

3    "ancestral-like" F1 segregants were pooled. These two DNA pools were

4    sequenced using an Illumina NextSeq500 system (Illumina, San Diego, CA)

5    with 150 bp paired-end reads from 350-bp libraries. At least 57X coverage was

6    achieved for sequencing ancestral and evolved clones, and we obtained

7    150-200X coverage for the pooled segregants. Sequence results were

8    analyzed using CLC Genomics Workbench 9.1 with default settings for read

9    import, read trimming, read alignment, duplicate-read removal, local

10   realignment, single nucleotide polymorphism (SNP) calling (with the slight

11   modification of using a 10% frequency cut-off in the "ancestral-like" pool to

12   retrieve as many mutations as possible since the default is 35%), and SNP

13   annotation. We used the reference genome of S288C (version R64-2-1) from

14   The *Saccharomyces* Genome Database for SNP calling because it is the best

15   annotated.

16

17   **Identifying candidate causal SNPs responsible for increased noise**

18   By using the CLC Genomics Workbench 9.1 function "filter by control read"

19   with a criterion of less than 2 control reads, we could eliminate SNPs existing

20   in both ancestral and evolved lines (Table S1). The resulting list of evolved

21   SNPs was then cross-referenced with the list of SNPs from the "evolved-like"

22   pool. We anticipated that candidate causal SNPs responsible for increased

23   noise would be enriched in the "evolved-like" but not in the "ancestral-like"

24   pools. By simulating sequencing results (using Python 2.7) from 150x

25   coverage, we determined the frequencies of SNPs that were randomly

1  segregated into two pools of 10 or 20 individuals to represent SNP enrichment

2  thresholds for increased noise [84]. We identified SNPs with a frequency >70%

3  in the "evolved-like" pool and with a frequency <32% in the "ancestral-like" pool

4  at a 90% confidence interval as being correlated with increased noise (Table

5  S2). The thresholds are represented by 90% confidence intervals for

6  simulations with 10 individuals and at 95% for those with 20 individuals.

7

8  **Histone chromatin immunoprecipitation (ChIP) combined with**

9  **Quantitative PCR (Q-PCR)**

10  Log-phase cells (~30 $OD_{600}$) were harvested and crosslinked by 1%

11  formaldehyde at 30 °C for 30 min. After quenching with 125 mM glycine, the

12  cell suspension was lysed by three cycles of 5 min beating and 1 min cooling

13  at 4 °C of 0.5 mm glass beads in FA buffer (50 mM HEPES-KOH pH=7.5, 140

14  mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.1% Deoxycholate Na salt)

15  supplemented with 1x protease inhibitor cocktail (Protease inhibitor cocktail

16  Set IV in DMSO; Merck, 539136) and 1 mM PMSF. We made a hole in the

17  bottom of the lysate-containing tube to allow the lysate to flow through into a

18  collection tube upon centrifugation at 500 x $g$ for 3 min at 4 °C. The chromatin

19  fraction was pelleted down by centrifugation at 12000 x $g$ for 15 min at 4 °C

20  and washed twice with FA buffer. We transferred 2 ml of the chromatin

21  suspension into a 15-ml centrifugation tube (BIOFIL), avoiding bubbles. We

22  sheared the chromatin using a Bioruptor (Diagenode, Denville, NJ), with 5

23  cycles of 15 min (30 sec On and 30 sec Off per min) at "High intensity" mode at

24  4 °C. Ice-cold water was resupplied in the Bioruptor tank after each cycle to

25  maintain the low temperature. The sheared chromatin was cleared by

26

1 centrifugation at 12000 x $g$ for 15 min at 4 °C. One-fifteenth of the cleared

2 chromatin was used as input and stored at -80 °C. The remaining cleared

3 chromatin was transferred into a tube containing pre-incubated Dynabeads®

4 Protein A (#10002D, Invitrogen, Waltham, MA) with histone 3 antibody

5 (ab#1791, Abcam), followed by end-over-end mixing overnight at 4 °C. The

6 beads were anchored with DynaMag™-2 Magnet (ThermoFisher Scientific,

7 Waltham, MA) to remove unbound molecules and were then sequentially

8 washed with 1 ml of FA buffer, 500 mM NaCl-containing FA buffer, DOC buffer

9 (10 mM Tris-HCl, 1 mM $Na_2EDTA$ pH=8.0, 250 mM LiCl, 0.5% NP-40, 0.5%

10 Deoxycholate Na salt), and TE buffer (10 mM Tris·Cl; 1 mM $Na_2EDTA$ pH=8.0).

11 The bound molecules were eluted by adding TES buffer (10 mM Tris·Cl; 1 mM

12 $Na_2EDTA$ pH=8.0, and 1% SDS) and incubating at 65 °C for 20 min, followed

13 by a second elution with TE buffer (65 °C for 10 min). The two eluents were

14 combined. The eluent and thawed input sample were incubated in 0.125 μg/ml

15 RNase A at 37 °C for 30 min, followed by Proteinase K treatment (2 mg/ml

16 Proteinase K at 42 °C for 1 h). The samples were de-crosslinked at 65 °C

17 overnight. DNA was purified using a QIAquick DNA Purification Kit (Qiagen,

18 Hilden, Germany) according to the manufacturer's instructions, with the

19 modification of a two-cycle wash step using Qiagen PE buffer. We subjected

20 0.5 ng of DNA to Q-PCR with primers annealing to nucleosome-occupied and

21 -depleted regions of the promoter and coding regions for *TDH2* and *GLY1*

22 (Table S3) [85].

23

24 **Q-PCR of mRNA under different growth conditions**

25 For stress conditions, log-phase cells grown in YPD media were divided into

1    four parts. One part was continuously grown in YPD as an untreated sample

2    and the remaining three parts were treated with 0.375 M KCl for 20 min or 0.4

3    mM $H_2O_2$ for 20 min or subjected to 42 °C for 30 min. For different growth

4    states, total RNA was extracted from the same batch of cultures when cells

5    were in log phase and entering diauxic shift. To investigate the activity of

6    SWI/SNF chromatin remodeling complexes, cells were first grown to log phase

7    in CSM-Serine (Serine-depleted Complete Synthetic Mixture). Then L-Serine

8    was added to a concentration of 0.1% to induce *CHA1* expression, before

9    harvesting cultures 0, 5, and 15 min after induction, with the addition of 0.05%

10   $NaN_3$ to stop transcription [49].

11       RNA from 5-10 $OD_{600}$ of cells was extracted and quantified according to a

12   previous report [68] with modifications. Cells were harvested at 4 °C. RNA

13   quality was examined using an Agilent RNA 6000 Nano kit with an Agilent

14   2100 Bioanalyzer (Agilent Technologies). Two μg of RNA was

15   reverse-transcribed with 0.5 μg oligo(dT)$_{18}$ primer using an Applied

16   Biosystem™ High-Capacity cDNA Reverse Transcription kit (ThermoFisher

17   Scientific). One μl of the reaction products was then used for Q-PCR in an

18   Applied Biosystem™ 7500 Fast Real-Time PCR system (ThermoFisher

19   Scientific) with gene-specific primers (Table S3).

20

21   **Statistical analyses**

22   All statistics were performed using R language (http://www.r-project.org/).

23

24   **Acknowledgements**

5

6  **Figure Legends**

7  **Fig. 1. Yeast cells exhibit high noise levels upon experimental evolution.**

8  (A) Schematic of our evolution experiment. Cells were treated with 2.8% EMS,

9  regrown for 12 h, and then selected for the top 5% (or bottom 5%) of the

10  population in terms of GFP intensity. Alternating selection enriched for mutant

11  cells that presented higher expression noise in terms of GFP intensity. (B)

12  Most of the evolved clones exhibit increased expression noise. Single clones

13  were isolated from eight evolved cultures and their reporter gene expression

14  was measured. The x- and y- axes represent fold-change in noise and mean

15  expression, respectively, after evolving. The median and range for 4-5

16  replicates for each evolved clone are indicated by circles and error bars,

17  respectively. The red lines indicate values for the ancestral line.

18

19  **Fig. 2. Cells with different levels of Tdh2-GFP exhibit different**

20  **physiological states.**

21  (A) Stationary-phase cells with high Tdh2-GFP levels (red circles) survive

22  better than those with low levels (blue squares) after growing in

23  $H_2O_2$-containing medium (Fisher's exact test, $p$ = 0.1099 for untreated

24  samples, $p$ = 1.127 × 10$^{-4}$ for $H_2O_2$-treated samples). Single cells with high or

1  low GFP intensities were sorted and plated on the same plates with or without

2  4.4 mM $H_2O_2$. Survival rates were determined by counting colony-forming units

3  after 5 days of growth. (B) Stationary-phase cells with high Tdh2-GFP tend to

4  re-enter the cell cycle faster than those with low Tdh2-GFP signal. Each dot of

5  the scatter plot represents data from a single cell. Unsorted stationary-phase

6  cells were placed on YPD agarose pads and only unbudded cells were

7  monitored using time-lapse microscopy. The x-axis indicates the initial

8  Tdh2-GFP signal intensity for each cell and the y-axis indicates the first

9  rebudding time. The red dotted line represents a linear regression

10  (Spearman's rank correlation, n=111, $p$ = 8.131 × $10^{-12}$). (C) Stationary-phase

11  cells with low Tdh2-GFP signal survive better than those with high signal when

12  cells encounter heat stress after being re-fed with fresh nutrients (one-sided

13  Wilcoxon rank-sum test, n=4-5; $p$ = 0.1995 for untreated samples, $p$ = 0.2426

14  for heat-treated samples before nutrient refreshment, $p$ = 0.0159 for

15  heat-treated samples after nutrient refreshment). Survival rates were

16  determined by counting colony-forming units after 3 days of growth. The

17  median value for replicates is indicated by solid horizontal lines among groups

18  of data points. *$p$ < 0.05, ***$p$ < 0.001.

19

20  **Fig. 3. Multiple genes in the evolved cells exhibit increased noise levels.**

21  (A) Four reporter genes (*TDH3-BFP*, *PGK1-BFP*, *ADK1-GFP* and *GLY1-BFP*)

22  were engineered in modified ancestral (red circles) and evolved (blue squares)

23  *TDH2-GFP*-carrying cells (see Materials and Methods), and their expression

24  noise was measured (one-sided Wilcoxon rank-sum test, n=7-8; $p$ = 4.534 ×

25  $10^{-4}$ for *ADK1*, $p$ = 0.0027 for *GLY1*, $p$ = 9.441 × $10^{-4}$ for *PGK1*, $p$ = 7.158 ×

1    $10^{-4}$ for *TDH3*). *TDH3* and *PGK1* are involved in *TDH2*-related pathways,

2    whereas *ADK1* and *GLY1* are not. (B) Transcriptional regulation is responsible

3    for increased noise in the evolved cells (one-sided Wilcoxon rank-sum test,

4    n=7-8; *p* = 4.495 × $10^{-4}$). The *TDH2* promoter was directly fused with the

5    coding sequence of *GFP*. This construct was engineered in modified ancestral

6    and evolved *TDH2-GFP*-carrying cells and its expression noise was

7    measured. The median value of replicates is indicated by horizontal solid lines

8    among groups of data points. **$p$ < 0.01, ***$p$ < 0.001..

9

10    **Fig. 4. The *hmt1-G70D* mutant recapitulates the increased noise**

11    **phenotype observed in the evolved line.**

12    **(**A) Reconstituting the *hmt1-G70D* mutation in the ancestral background

13    increases Tdh2-GFP noise, whereas reversing that mutation in the evolved

14    background decreases it (one-sided Wilcoxon rank-sum test, n=6; *p* = 0.0011

15    for the ancestral background, *p* = 0.0025 for the evolved background). (B)

16    Reconstituted *hmt1-G70D* mutant cells exhibit increased expression noise of

17    the *TDH2* promoter-GFP construct (one-sided Wilcoxon rank-sum test, n=8; *p*

18    = 0.0002). (C) Reconstituted *hmt1-G70D* mutant cells present increased noisy

19    expression of Adk1-GFP, Gly1-BFP, and Tdh3-BFP (one-sided Wilcoxon

20    rank-sum test, n=7-8; *p* = 0.0030 for *ADK1*, *p* = 0.0011 for *GLY1*, *p* = 0.0030

21    for *TDH3*). All mutants were constructed using the CRISPR/Cas9 system. The

22    median value of replicates is represented by horizontal solid lines among

23    groups of data points. **$p$ < 0.01, ***$p$ < 0.001.

24

1    **Fig. 5. Mutations of SWI/SNF components and the small ribosomal**

2    **subunit Rps2 result in elevated noise.**

3    (A) Attenuating *RPS2*, *SNF2*, or *SNF5* gene expression in the ancestral

4    background results in increased noise (one-sided Wilcoxon rank-sum test,

5    n=6-19; *p* = 0.0039 for *rps2*-DAmP/+, *p* = 0.0025 for *snf2Δ/+*, *p* = 0.0008 for

6    *snf5Δ/+*). Rps2 and Snf2 are methylation substrates of Hmt1. Snf2, Snf5, and

7    Snf6 are essential components of the SWI/SNF chromatin remodeler. Since

8    haploid mutants of *rps2*-DAmP, *snf2Δ*, and *snf5Δ* exhibit severe growth

9    defects and as slow growth has been shown to increase expression noise [86],

10   we constructed heterozygous mutant diploids to measure noise. (B) Deletions

11   of *NPL3* or *SBP1* in haploid cells do not result in increased noise (one-sided

12   Wilcoxon rank-sum test, n=5; *p* = 0.0079 for *npl3Δ*, *p* = 0.206 for *sbp1Δ*). Npl3

13   and Sbp1 are methylation substrates of Hmt1. (C) Deleting *SNF6* in the

14   ancestral background results in increased noise (one-sided Wilcoxon

15   rank-sum test, n=5; *p* = 0.0087). Haploid *snf6Δ* mutants exhibit defective

16   respiration, so we used a haploid rho- ancestral strain in this experiment and

17   cells were grown in YPD medium. The median value of replicates is

18   represented by horizontal solid lines among groups of data points. (D) The

19   activity of SWI/SNF chromatin remodeling complexes is compromised in the

20   *hmt1-G70D* mutant. It has been shown previously that compromising SWI/SNF

21   complexes results in attenuated transcriptional activation of *CHA1* [49]. Total

22   RNA was isolated from ancestral, *hmt1-G70D,* and *snf5Δ* haploid cells 0, 5,

23   and 15 min after adding 0.1% L-Serine (an inducer of *CHA1* expression).

24   Specific mRNA levels were assessed by Q-PCR. In the figure, *CHA1* mRNA

25   levels were normalized to those of *PYK1*, with this latter acting as an internal

1   control. For all time-points, *CHA1* mRNA levels in ancestral cells (blue) were

2   significantly higher than those in *hmt1-G70D* (red) or *snf5Δ* (green) mutant

3   cells (one-sided Student's t-test, n = 9; *p* < 0.01). (E) *hmt1-G70D* mutant cells

4   display higher nucleosome occupancy in the promoter and partial coding

5   regions of *TDH2* and *GLY1*. The chromatin status of the *TDH2* promoter was

6   established by chromatin immunoprecipitation against histone 3 coupled with

7   Q-PCR. The numbers in the x-axis indicate the distance (in bp) from the

8   transcription start site. The y-axis represents relative enrichment of histone 3

9   signals for the amplicons at the indicated regions (one-sided Student's t-test, n

10  = 10-12). Black, grey, and dashed circles indicate confirmed, fuzzy, and

11  condition-specific nucleosome-occupied regions, respectively. Error bars

12  represent standards errors. *$p$ < 0.05, **$p$ < 0.01, ***$p$ < 0.001.

13

14  **Fig. 6. Hmt1-mediated noise suppression can be released under**

15  **non-optimal growth conditions as a conserved cell survival strategy.**

16  (A) *HMT1* transcripts are down-regulated under non-optimal growth conditions

17  (one-sided paired Student's t-test, n = 4; *p* = 0.0008 for heat stress, *p* = 0.003

18  for oxidative stress, *p* = 0.002 for osmotic stress, *p* = 0.007 for diauxic shift).

19  The level of mRNA was measured using Q-PCR, and *DET1* mRNA was used

20  as the internal control. Error bars indicate standard errors. (B) Non-optimal

21  growth conditions result in increased Tdh2-GFP noise (one-sided Wilcoxon

22  rank-sum test, n = 4; *p* = 0.015 for heat, oxidative and osmotic stress, *p* =

23  0.014 for diauxic shift). Noise was measured 2.5 h after cells had been shifted

24  to the indicated conditions or was measured at different growth stages. (C)

25  *hmt1-G70D* mutant populations survive better than the ancestral line in

1    medium containing $H_2O_2$ (Fisher's exact test; $p < 2.2$ x $10^{-16}$). Stationary-phase

2    cells were spread on plates with 5.3 mM $H_2O_2$ and survival rates were

3    determined by counting colony-forming units after 5 days of growth. (D)

4    Mutation of the *HMT1* ortholog in the fission yeast *S. pombe* also results in

5    increased expression noise. *rmt1* is the ortholog of *HMT1*, whereas *tdh1* and

6    *gpd3* are *TDH2* orthologs. Deletion of *rmt1* increased protein noise of both

7    tdh1-YFP and gpd3-YFP (one-sided Wilcoxon rank-sum test, n = 10-12; *p* =

8    0.0005 for *tdh1-YFP*, *p* = 0.037 for *gpd3-YFP*). The median value of replicates

9    is indicated with horizontal solid lines among groups of data points. **p* < 0.05,

10   ***p* < 0.01, ****p* < 0.001.

11

12   **Fig. 7. A model showing how Hmt1 modulates cell-to-cell heterogeneity**

13   **in response to environmental stress.**

14   Hmt1 methylates and enhances the function of the SWI/SNF chromatin

15   remodeler and small ribosomal subunits to reduce stochastic noise in gene

16   expression. Under normal conditions, cells maintain a high level of Hmt1 and

17   exhibit homogeneous gene expression in most cells. However, when the

18   population encounters environmental stress, *HMT1* expression is

19   down-regulated, inhibiting the functions of Hmt1 targets. Accordingly,

20   expression of Hmt1 gene targets becomes noisier so individual cells exhibit

21   heterogeneous cell physiologies. The likelihood of population survival is

22   enhanced due to this heterogeneity.

23

24   **Supplemental Figure Legends**

**Fig. S1. Sorted subpopulations of cells exhibiting low or high Tdh2-GFP are not genetically distinct.**

Cells were sorted according to their Tdh2-GFP levels into low (red circle) and high (blue circle) subpopulations, each of which constituted 10% of the whole population (grey). These cells were grown in YPD for the indicated periods of time and analyzed by flow cytometry. The distributions of the low (red) and high (blue) Tdh2-GFP subpopulations are superimposed with that of the parental population (grey outline). The vertical dashed lines indicate the mean signal intensity of the parental population.

**Fig. S2. Experimental evolution alters the Tdh2-GFP signal distribution without affecting protein integrity or subcellular localization.**

(A) Increased noise in the evolved Tdh2-GFP-carrying line is due to a flattened distribution rather than a bimodal one. (B) Full-length Tdh2-GFP is retained after evolution. Western blots were hybridized using mouse anti–GFP antibody (1:4000) (the upper blot) and by rabbit anti–G6PDH antibody (1:4000) (the lower blot). (C) Protein localization of Tdh2-GFP is not altered by our experimental evolution approach. Cells were imaged using a 60x objective under the FITC channel (the upper panel) or bright field (the lower panel). The scale bar is 5 $\mu$m.

**Fig. S3. Tdh2-GFP signal in F1 progeny selected for whole genome sequencing.**

1 For bulk segregant analysis, 360 F1 progeny were derived by backcrossing the

2 evolved clone to the ancestral clone. The noise and mean of Tdh2-GFP signal

3 in individual progeny were analyzed, and a total of three runs of noise

4 measurement were conducted to identify "evolved-like" and "ancestral-like" F1

5 segregants. Data from the final run of analysis are shown here. gDNA of the

6 "evolved-like" and "ancestral-like" F1 progeny was extracted and respectively

7 pooled for whole genome sequencing.

8

9 **Fig. S4. The *hmt1-G70D* mutation phenocopies the loss-of-function**

10 **mutation.**

11 (A) The identified G70D mutation of Hmt1 is located in a highly conserved

12 methyltransferase motif. An alignment of the primary sequences of the

13 conserved motif is shown for various methyltransferases from budding yeast,

14 fission yeast, human and bacteria. Residues shared with *S. cerevisiae* Hmt1

15 are labeled in yellow, and the mutated glycine residue observed in the evolved

16 clone is indicated by an arrowhead. (B) Both *hmt1-G70D* and Hmt1 deletion

17 mutants exhibit a similar level of increased Tdh2-GFP noise (one-sided

18 Wilcoxon rank-sum test, n=5-10; *p* = 0.0013 for *hmt1-G70D*, *p* = 0.0013 for

19 *hmt1△*). (C) The G70D mutation of Hmt1 results in defective methylation. The

20 patterns of asymmetric di-methylation (left) and pan-methylation (right) of

21 arginine in whole cell lysates of *hmt1-G70D* mutant cells did not differ from

22 those of deletion mutants (*hmt1△*), but differed from those of wild type cells.

23

24 **Fig. S5. Tdh2-GFP noise significantly increases shortly after stress**

1    **treatments.**

2    Tdh2-GFP noise increases under non-optimal growth conditions (one-sided

3    Wilcoxon rank-sum test, n=4; *p* = 0.015 for heat stress, *p* = 0.015 for oxidative

4    stress). Noise was measured after cells were treated with the indicated stress

5    for 20-30 min. The difference between untreated and treated cells became

6    more obvious after 2 h (Fig. 6B), which probably reflects the time it takes for

7    cells to alter the abundance of Tdh2-GFP protein. The median value of

8    replicates is indicated with horizontal solid lines among groups of data points.

9    *\*p* < 0.05.

10

11    **Supplemental Tables**

12    **Table S1. Mutations in the evolved *TDH2-GFP-carrying* strain.**

13    **Table S2 Identities and frequencies of candidate mutations responsible**

14    **for increased noise, and identified mutations in the ancestral-like and**

15    **evolved-like F1 progeny pools.**

16    **Table S3. Plasmids and primers used in this study.**

17

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# Figure 1

## A

### Reporter gene construct



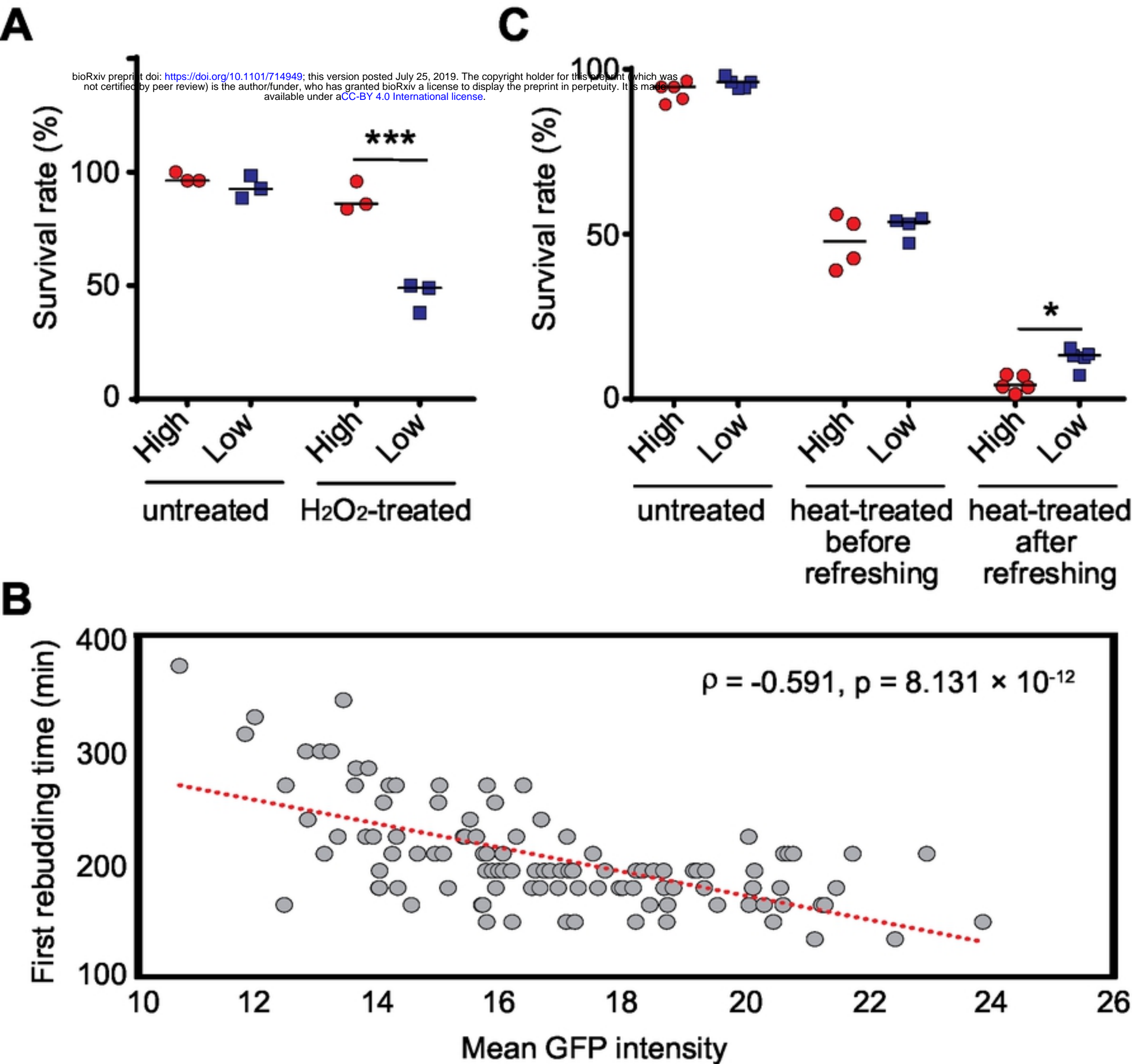### Experimental evolution

## B



Figure 1

# Figure 2

**A**

Survival rate (%)

High — Low  High — Low
untreated   H$_2$O$_2$-treated

***

**C**

Survival rate (%)

High — Low  High — Low  High — Low
untreated   heat-treated   heat-treated
            before         after
            refreshing     refreshing

*

**B**

First rebudding time (min)

$\rho = -0.591, p = 8.131 \times 10^{-12}$

Mean GFP intensity

Figure 2

# Figure 3

**A**



ADK1-GFP

GLY1-BFP

PGK1-BFP

TDH3-BFP

**B** TDH2 promoter-GFP

Figure 3

# Figure 4

## A
### TDH2-GFP



## B
### TDH2 promoter-GFP
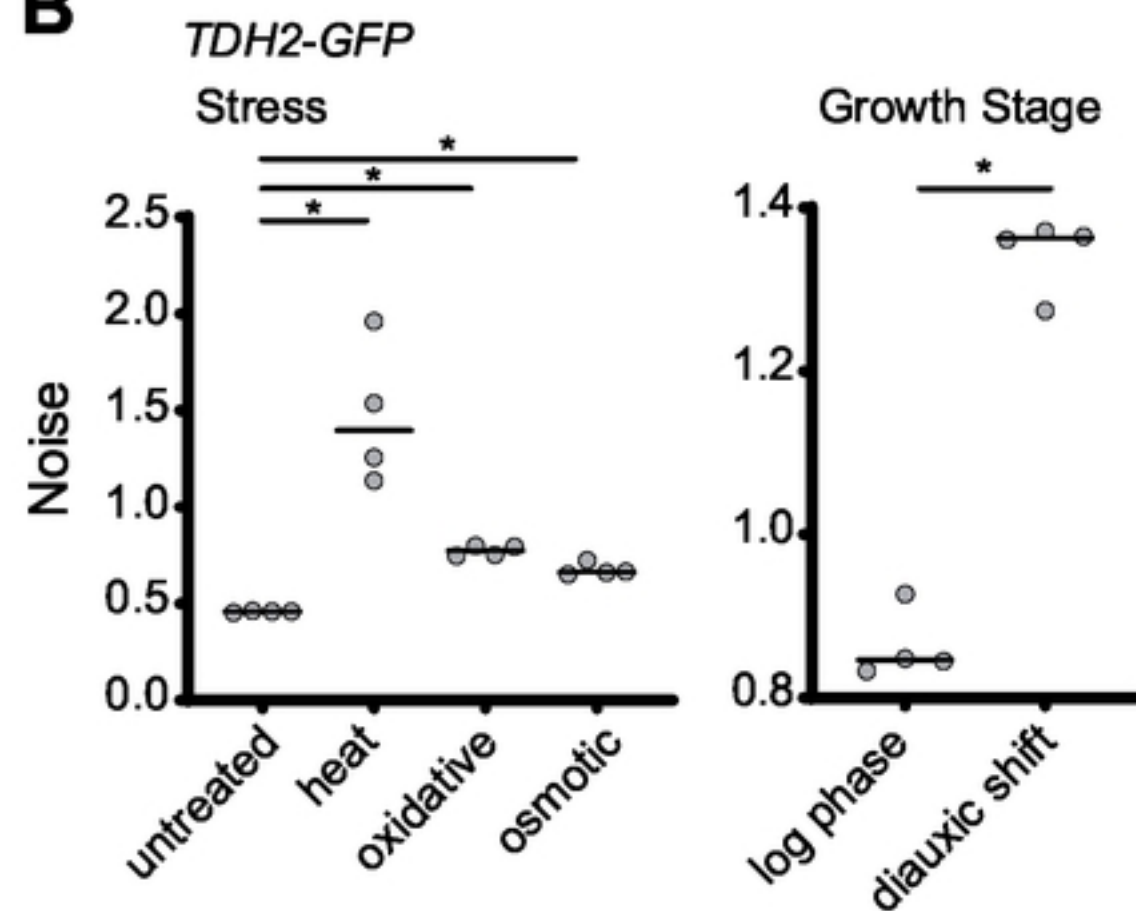
## C

### ADK1-GFP

### GLY1-BFP

### TDH3-BFP



Figure 4

# Figure 5

Figure 5

Figure 6



Figure 6

# Figure 7

Figure 7