Protein level variability determines phenotypic heterogeneity in proteotoxic stress response

Marie Guilbert, François Anquez, Alexandra Pruvost, Quentin Thommen and Emmanuel Courtade

UMR 8523, PhLAM – Physique des Lasers Atomes et Molécules, CNRS, Université de Lille, France

Keywords
- cellular heterogeneity; heat shock response; mathematical modeling; signaling dynamics; single-cell analysis

Correspondence
Q. Thommen, UMR 8523 – PhLAM – Physique des Lasers Atomes et Molécules, CNRS, Université de Lille, Lille F-59000, France
Tel: +33 320 337 018
E-mail: quentin.thommen@univ-lille.fr
(Received 21 August 2019, revised 3 February 2020, accepted 16 March 2020)
doi:10.1111/febs.15297

Introduction

Resistance of a population subfraction to a cancer treatment (chemotherapy, for instance) limits the effectiveness of the treatment [1] and is named cellular response heterogeneity. Obviously, extracellular environment variations or genetic alterations induce cellular heterogeneity in treatment response, but several massive single-cell experimental results [2-8] reveal that a significant phenotypic heterogeneity persists even for monoclonal cell lines and in a uniform environment. The discovery of the underlying molecular mechanisms leading to variability in response to treatment and their potential control is a major issue for cancer therapy research [9,10].

In a clonal cell line, intracellular biochemical fluctuations result in a cell population with the same genome but with various proteomes [11,12]. Cell-to-cell variability can arise from such intracellular biochemical fluctuations and is called nongenetic heterogeneity (NGH) [13]. NGH plays a functional role in surviving unpredictable environmental changes [11,14,15], and it has been identified in anticancer treatment as an inducer of fractional killing [16-18]. Accurate clinical models including NGH are still to be built in order to guide diagnosis and treatment of diseases [19]. Indeed, the precise knowledge of the molecular network is not enough to predict the response of a cell population to a given treatment. Such models would also require the identification of the key molecular players [20,21] and a detailed study of NGH [22,23].

Abbreviations
- CCRV, cell-to-cell response variability; HSC70, heat shock 70-kilodalton protein 8 (HSPA8); HSE, heat shock element; HSF1, heat shock factor 1; HSP, heat shock protein; HSP70, 70-kilodalton heat shock proteins family; HSP72, heat shock 70-kilodalton protein 1 (HSPA1A); HSRN, heat shock response network; NGH, nongenetic heterogeneity; nSB, nuclear stress body.
A general feature of cellular stress response networks is the response-to-stimuli ultrasensitivity: The response increases slowly at low stimulus value, and it sharply increases to a high value once a given stimulus threshold is reached. Ultrasensitivity is well known to arise from networks having either a positive cooperativity, multi-step processes, or protein sequestration [24, 25]. Such architectures tend to shrink cell-to-cell response variability (CCRV) due to NGH for low or moderate stress strengths. In contrast, the response-to-stimuli ultrasensitivity tends to broaden CCRV once the stimulus approaches the threshold value. Stress response-ultrasensitive networks are thus appropriate to study the cellular heterogeneity arising from NGH because ultrasensitivity acts as a heterogeneity amplifier.

The heat shock response network (HSRN) in the cytosol, together with the unfolded protein response in the endoplasmic reticulum, is essential for maintaining the proteome integrity [26]. HSRN displays ultrasensitivity due to protein sequestration mechanism [25, 27]. Several proteotoxic stresses, such as oxidation or heat, trigger HSRN, which induces the transcription of heat shock proteins (HSPs) via activation of heat shock factor 1 (HSF1) transcription factor. HSPs act as molecular chaperones to maintain cellular proteostasis [28].

At the single-cell level, HSF1 forms dynamic structures named nuclear stress bodies (nSBs) [29]. In the present study, we first show that nSBs can be used to quantify HSRN activation at the single-cell level. We then use high-throughput time-lapse microscopy with a precise control of hyperthermia temporal profile (41–43 °C) to monitor nSBs dynamics in a monoclonal population. From computational image analysis, large datasets of quantitative single-cell nSBs temporal dynamics have been constructed several hours after heat shock. These data allow us to shed light on an unexpected broad range of response to a given stimulus. We then address the molecular underpinnings of such CCRV in HSRN dynamics. Response variability is investigated by both statistical analysis of data and network parameter sensitivity analysis of a data-driven mathematical description of the HSRN. Using computational prediction and experimental characterization of single cells, we finally identify NGH to play a crucial role in CCRV by modulating HSF1 and HSP concentrations across the cell population.

Results

Monitoring HSF1 activation of individual HeLa cells

Under normal conditions, the molecular chaperone heat shock protein 70 kDa (HSP70) sequesters HSF1. Under stressful conditions, HSF1 is unbound from HSP70 [30, 31]. Free HSF1 (i.e., relaxed from HSP70) can form homotrimers [32, 33] and can bind on a specific region of DNA named heat shock elements (HSE). HSF1 bound to HSE promotes the transcription of the wide family of HSP proteins that includes HSP70 and others [34–38]. In human and primate cells, free HSF1 also forms nuclear stress bodies (nSBs, also named as HSF1 foci), which are reversible macromolecular complexes made of (among other macromolecules) HSF1 trimers bound to heterochromatin regions without HSE [29]. If HSF1:eGFP binding to HSE provides an insufficient fluorescent signal to be efficiently monitored using conventional fluorescence microscopy, a single nSB does [29, 37]. nSBs are formed within seconds under stressful conditions [29] and form foci in the cell nucleus that can be observed under a conventional fluorescence microscope (Fig. 1A and [37]). The quantity of HSF1:eGFP within foci can be measured over statistically significant cell population by using an automated images analysis. We define F as the fraction of HSF1:eGFP fluorescence signal located in foci to measure HSR activation for an individual cell. F is a ratiometric measurement proportional to the fraction of HSF1 free from HSP70. F provides a readout of HSF1 activation in individual cells (Fig. 1A).

In order to compare our single-cell method with conventional biochemistry measurements, we first study the dynamics of F averaged over the whole-cell population upon a temperature rising up from 37 °C to 41 °C, 42 °C, or 43 °C. As shown by Abravaya et al. [30], dynamics of HSF1 bound to HSE at these three temperatures deliver the big pictures of the HSR dynamics upon heat stress. Indeed, the dynamics of activated HSF1 at 42 °C is drastically different from the one at 43 °C: At the former temperature, activated HSF1 exhibits pseudo-adaptation kinetics, while at the later temperature, HSR activation persists (Fig. 1B). The time evolution of F average over the whole population is in very good agreement with biochemical measurements for all three temperatures (Fig. 1C). The genetic modification resulting from the HSF1:eGFP insertion does not impact the F kinetic, as revealed by time point immunofluorescence staining measurement in wild-type HeLa (HeLa WT) cell line (Fig. 2A). We conclude that HSF1 foci dynamic represents a valid reporter of the HSRN activation upon heat shock.

High-throughput screening of HSRN reveals broad cell-to-cell variability

Averaging over the cell population gives at first glance a misconception: a population of cells all having similar

5346 The FEBS Journal 287 (2020) 5345–5361 © 2020 Federation of European Biochemical Societies
foci intensity whose brightness increases with stress amplitude. Examining the time traces of individual cells with identical genome and exposed to the exact same stimulus reveals that this picture is not correct. One easily distinguishes a broad cellular heterogeneity both in foci intensity and dynamics (Fig. 3A–C). These experimental results were confirmed using a second monoclonal cell line (Fig. 4). Besides, no spatial dependency was found for the amplitude neither the shape of the response. This confirms that heterogeneity is not due to a spatial distribution of the temperature across the sample nor any other imaging artifact. Although HSF1:eGFP expression level varies significantly from cell to cell, the total HSF1:eGFP amount in a single cell does not vary during the experiment and then does not impact the foci dynamics (Fig. 3D–F).

If we now focus on the response at a given time (1 h after the stress onset), we observe a significant fraction of cells that do not display detectable foci (78.4% at 41 °C, 50.5% at 42 °C, and 19.7% at 43 °C), while the responding subset displays a wide distribution of free HSF1 with $F$ values up to 0.5

---

**Fig. 1.** Screening of foci dynamics in individual HeLa cells. (A) Example of foci dynamics in an individual cell. During one experiment, around 300 individual cells are tracked, and experiments are performed in duplicate and confirmed with another HeLa cell line transfected with HSF1:eGFP construct. Upper panel: snapshots of single HeLa HSF1:eGFP cell over time upon a 42 °C heat stress; in the images, the white scale bars correspond to 10 μm; middle panel: dynamics of the fluorescence intensity measured in the center of the three visible foci (crosses, circles, and stars) and average fluorescence level over the entire cell nucleus (dots); bottom panel: dynamics of the fraction of HSF1:eGFP fluorescence within foci $F$. (B) Dynamics of activated HSF1 as measured by [30] upon a 41 °C (black), 42 °C (orange), and 43 °C (red) heat stress by run on assay. (C) Dynamics of the faction of HSF1:eGFP fluorescence ($F$) in nSBs monitored over time average over the whole-cell populations upon a 41 °C (black), 42 °C (orange), and 43 °C (red) heat stress for a large cell colony; dots stand for average values with error bars on the side (standard deviation). In all cases, time zero coincides with the stress onset.

**Fig. 2.** Foci intensity dynamic in wild-type cell line during a 43 °C heat stress. (A) Averaged value over the cell population of foci intensity measured on a single cell (dots) and associated error bars. (B) Foci intensity in a single cell ordered by increasing value. In both pictures, the color code is as follows: yellow—37 °C; red—half an hour after the onset of a 43 °C; orange—1 h after the onset of a 43 °C; and black—3 h after the onset of a 43 °C.
These results suggest that the rise of response amplitude with increasing temperature observed at the population level (Fig. 1B,C) is at least partially due to an increase in the fraction of responding cells rather than solely due to an absolute increase in free HSF1 fraction per cell. Similar results are obtained with HeLa WT cell line (Fig. 2B).

The temporal shape of the response also varies across the cell population. We observe cells exhibiting complete relaxation and cells with $F$ monotonously increasing. To capture the heterogeneity of HSF1 activation dynamics, we define a relaxation index $\eta$ as the ratio of the response at 1 h to the one at 3 h after heat shock (Fig. 3H). $\eta = 0$ indicates a near-perfect adaptation; $\eta = 1$ translates into a plateau; and $\eta > 1$ is a sign of continuously increasing activation. It is worth noting that some cells exposed to step temperature increase at 42 °C show dynamics comparable to the population average at 43 °C and vice versa. Indeed, 7.5% of the responding cells have a relaxation index $\eta \geq 0.75$ for 42 °C heat shock, whereas 12% of the responding cells show a relaxation index $\eta \leq 0.75$ at 43 °C. Finally, we note that the temporal shape of the response is not correlated with the $F$ value 1 h after the stress onset (Fig. 3I).

**Variation in protein basal expression level can induce heterogeneous cellular response**

One surprising feature of our single-cell dataset is the apparently continuously varying behavior across the cell population (Fig. 3A–C). Our attempts to apply statistical clustering methods to each dataset could not

![Fig. 3. Cell-to-cell variability in heat shock response. The HSF1:eGFP fluorescence and fraction of HSF1:eGFP fluorescence in nSBs (F) are monitored over time in a single cell upon a 41 °C, 42 °C, and 43 °C heat stress; time zero coincides with the stress onset. (A–C) Cell temperature–time profile (upper panel) and $F$ as a function of time in a single cell (lower panel); in the color image, each horizontal line corresponds to a single cell, and the color code indicates the $F$ value measured at a given time, according to the scale bar on the right. (A) 41 °C; (B) 42 °C; and (C) 43 °C. (D–F) HSF1:eGFP fluorescence as a function of time in a single cell is monitored over time in a single cell. Each horizontal line corresponds to a single cell, and the color code indicates the HSF1 fluorescence measured at a given time, according to the scale bar on the right. (D) 41 °C; (E) 42 °C; and (F) 43 °C. (G) Distribution of $F$ across the cell population for several heat shock temperatures 1 h after the stress onset (cell ranking is similar to A–C). (H) Statistical distribution of the relaxation index defined as the ratio of the foci intensity measured in a given cell at three hours after the stress onset to the one measured 1 h after the stress onset. (I) Correlation between $F$ value one hour after the stress onset and the relaxation index.](image-url)
converge toward a finite number of phenotypes. A situation with only two clusters corresponding to the responding cells, on the one hand, and undetectable responses, on the other hand, is not satisfying as it would hide heterogeneity in the former class. We concluded that the variety of kinetic traces could not be captured by a discrete set of typical behaviors. At the network level, HSR is characterized by two competitive sequestration mechanisms. The output of such motif is known to be highly sensitive to protein concentration [24,25]. To explain the observed heterogeneity, we hypothesized that a variation in basal protein expression level across cell population could lead to significant differences in cellular responses. Indeed, protein expression levels vary from one cell to another even in a monoclonal cell line. This can be due to the stochastic expression of the gene [12] or asymmetric cell division [39]. To assess this possibility and gain understanding on the origin of CCRV, we derived a coarse-grained mathematical model of the HSR network.

In a minimal description, HSRN involves three different species: (a) misfolded proteins (MFPs) that are heat-induced and (b) HSPs that help to refold MFPs and (c) HSF1s that promote transcription of HSPs. The dynamics of the network is mainly regulated by two complexes that both involve the chaperone HSP [27]: HSPs sequester the MFPs, on the one hand, and HSF1s on the other. Our model accounts for the temporal evolution of copy number of four molecular species (MFP, HSP mRNA, HSF1, and HSP). The model uses ordinary differential equations where the fast dynamics of molecular complex assembly and disassembly are adiabatically eliminated (see the Materials
and methods section for details). We also account for the measured temperature rise time of the incubator. The coarse-grained model is accurate enough to quantitatively describe the foci dynamics (Fig. 5).

Using the above-described mathematical model, we show that reducing or increasing by only twofold the basal HSP concentration is sufficient to qualitatively mimic the dynamics of $F_{Th}$ (Fig. 6A–C and D–F compared to Fig. 3A–C). In our mathematical framework, the response heterogeneity is captured as a consequence of protein copy number variability: The more is the HSP number, the less is the foci intensity ($\eta_{Th}$) and the lower is the relaxation index ($\gamma_{Th}$). Moreover, the population-level observations are also predicted: (a) Both $F_{Th}$ and $\eta_{Th}$ increase with temperature; (b) relaxation of foci intensity ($\eta_{Th} < 0.5$) is more likely to appear at 41 °C and 42 °C than at 43 °C; and (c) a plateau or a slow increase ($\gamma \geq 1$) is observed mostly at 43 °C. Similar in silico results were obtained by varying HSF1 concentration. We conclude that variations in both HSF1 and HSP expression levels could lead to the experimentally observed CCRV.

One of the major advantages of our model is that it provides an explicit analytical expression for the foci intensity $F_{Th}(t)$ at any time $t$ after the stress onset (Eqn 5). $F_{Th}(t)$ depends on the concentration of the three main molecular actors, namely HSP, HSF1, and MFP. Mapping $F_{Th}$ as a function of HSP and HSF1 concentrations reveals iso-response lines (Fig. 6G–I). Such a mapping can be used to test the theoretical prediction.

**HSP72 and HSC70 expression levels impact cellular response and lead to cell-to-cell heterogeneity of the HSR**

In silico results suggest that both HSP and HSF1 level variations may induce the observed cell-to-cell variability in response to heat stress. Therefore, in order to test our theoretical predictions, we use immunolabeling and fluorescence microscopy to measure simultaneously HSP and HSF1 concentrations together with the response $F$ at the single-cell level. Our model assumes a generic HSP while the HSP family is wide and comprises several variants with specific roles [40]. However, only the HSP70 subfamily appears to play a significant role in HSF1 titration and consequently in its activation [41]. We thus consider only two members of the HSP family: HSP72 and HSC70. Both proteins play a similar role in sequestrating HSF1 and the refolding of MFP [42], but HSP72 is a stress-inducible protein (the transcription rate of $hsp72$ mRNA increases with the free concentration of HSF1), whereas HSC70 is not stress-induced and constitutively expressed [43].

In a first step, we estimated HSF1, HSP72, and HSC70 concentrations in individual cells from single immunolabeling in both HeLa WT and in HeLa HSF1:eGFP cell lines. Experimental data were compared for two thermal conditions: without heat shock and 1 h after exposure to a temperature step-up from 37 °C to 43 °C (Fig. 7A–C). As HSF1 is located in the cell nucleus ([44] and Fig. 1A), we focus on nuclear concentration for all three proteins. We used Hoechst staining of cellular DNA to allow automated cell segmentation of the cell nucleus. All three protein concentrations at both 37 °C and 43 °C are well fitted by a lognormal distribution (see Fig. 8 and the Materials and methods section). As expected, only HSP72 exhibits a shift of the distribution upon heat stress (Fig. 7B). The HSF1:eGFP insertion induces an overexpression of both HSF1 and HSP72 (1.46 for HSF1 and 1.65 for HSP72) but has no effect on HSC70. HSF1:eGFP insertion also induces a broadening of the
HSF1 and HSP72 distributions, especially toward higher values for both species. In a second step, we quantify the influence of HSP and HSF1 expression levels on the response amplitude. To do so, in HeLa WT cells we estimate HSF1 and HSP72 (or HSC70) concentrations via double immunolabeling. One hour after exposure to a step temperature increase at 43 °C, we measure \( F \) for the whole-cell population from HSF1 immunolabel. We then compute the population average of \( F \) conditional to a given value of HSF1 and HSP72 immunofluorescence signals (Fig. 7D). In agreement with the model prediction (Fig. 6), \( \langle F \rangle \) increases with HSF1 level and decreases with HSP72 level. In contrast, no significant correlation is found between \( \langle F \rangle \) and HSC70 protein expression level (Fig. 7E).

As shown above (Fig. 7A–C), the HSF1:eGFP insertion increases the number of cells having higher concentration of HSF1 and HSP72. We perform HSP72 (or HSC70) immunolabeling in HeLa HSF1:eGFP 1 h after exposure to a temperature step-up from 37 °C to 43 °C to compute maps similar as in Fig. 7D,E with a stretched variability in protein distributions (Fig. 7F, G). In this case, we monitor HSF1:eGFP fluorescence to measure HSF1 expression level and \( F \). The stretched protein distribution makes the dependence of \( \langle F \rangle \) on HSP72 protein expression level even more obvious. Immunolabeling of HSC70 reveals a dependence of \( \langle F \rangle \) also on HSC70 concentration. Importantly, we note that the average \( F \) value is similar in HeLa WT and in HeLa HSF1:eGFP experiments for a given HSF1 and HSP expression level. The mapping of \( F \) conditional to HSF1 and HSP concentration in HeLa HSF1:eGFP cells allows us to monitor rare events in which HSF1 and HSP72 concentrations are higher.

**Foci intensity correlates with HSP/HSF1 and monitors the fraction of free HSP**

The mathematical expression of \( F_{th} \) (Eqn 5) suggests that the foci intensity correlates with the chaperone-to-HSF1 ratio. To test this hypothesis and to interpret immunofluorescence data displayed in Fig. 7, we first numerically synthesize a set of \( F_{th} \) values in the same condition (1 h after a 43 °C stress onset) from model Eqn (6) and lognormal distributions of HSP and HSF1. Lognormal distributions are chosen to have the same averages as model Eqn (3), and the same width as that measured by immunofluorescence (Fig. 7). Figure 9 compares the numerically synthesized data to immunofluorescence data targeting HSP72 or HSC70 in HeLa HSF1:eGFP cell lines already displayed in Fig. 7F,G. We focus on HeLa HSF1:eGFP cell line results because the protein distributions are broader,
and thus less sensitive to noise estimation of $F$. To help the comparison, HSP72, HSC70, and HSF1 fluorescence signals are rescaled so that HSP72 and HSC70 fluorescence averages are identical to numerical HSP averages (0.479 µM), and HSF1 fluorescence averages are identical to numerical HSF1 averages (0.04 µM).

The 2D histograms (Fig. 9A–C) reveal a similar correlation between $F$ (or $F_{Th}$) and the chaperone-to-HSF1 ratio. To highlight the correlations, we display on the same plot the mathematical function $F_a$ given by Eqn (1):

$$F_a = \left( \frac{1}{1 + \alpha x} \right)^3,$$

where $x$ stands for the chaperone-to-HSF1 ratio. The mathematical shape of $F_a$ comes directly from the $F_{Th}$ mathematical expression (Eqn 5). In each case, the $\alpha$-parameter value is chosen to minimize the interquartile range (IQR) of the residuals $F_{Th} - F_a$ or $F - F_a$ (Fig. 9D–F). From the foci intensity and the residual distributions (Fig. 9D–F), one can extract the percentage of $F$ (or $F_{Th}$) distribution explained by the chaperone-to-HSF1 ratio through $F_a$ dependency by using IQR. It turns out that Eqn (1), with constant $\alpha$ value, explains 58% of the numerically synthesized data $F_{Th}$ distribution (Fig. 9D) and 56% and 48% of the experimental $F$ distribution (Fig. 9E,F). Those results indicate a strong correlation between the foci and the chaperone-to-HSF1 ratio, as well as a strong similarity between numerically synthesized and experimental datasets.

It might be striking that even in numerically synthesized data, the 2D histograms do not perfectly fit with the $F_a$ function. In fact, at least in the synthesized data, $\alpha$ corresponds to the fraction of free HSP, and thus, $\alpha$ is not a constant parameter, but $\alpha$ varies with the HSP value. So, if it is assumed that $\alpha$ has the same meaning in the experimental data, one can recast the $F$ (or $F_{Th}$) data to extract the $\alpha$ distribution, which is the distribution of the fraction of free HSP (i.e., not bounded to MFP) (Fig. 9G–I). Therefore, the HSF1 foci may also be used as an indirect probe of the chaperone free fraction.

**HSP redundancy: A strategy to reduce the cell-to-cell variability?**

The continuum of foci response amplitudes and foci temporal dynamic, which characterize the observed CCRV, arises from two facts: (a) The sequestration mechanisms underlying the heat stress response induce
a high sensitivity to protein expression level and (b) the stochastic expression of genes induces protein expression level distribution. The heat shock protein 70 family contains numerous homologous chaperone proteins (at least height). HSP72 (the stress inductive chaperone) and HSC70 (the constitutive chaperone), the major members of the family, are lognormally distributed with a similar variance.

A question arises as to whether the HSP redundancy may be a strategy to reduce the cell-to-cell variability. To illustrate the phenomena, let us focus on the foci intensity 1 h after a 43 °C stress onset; assume a total pool of chaperone HSP to be built from two distinct genes and then to be the sum of two independent homologous lognormally distributed proteins, here named HSP$_a$ and HSP$_b$ (Fig. 10A,B); and monitor the foci intensity distribution when the relative abundance of the two independent proteins varies (Fig. 10D), going from a case of a large prevalence of one isoform (Fig. 10, yellow lines) to a case of two equal distributions (Fig. 10D, black lines). In practice, the foci intensity is computed via mathematical model Eqn (6) with $\langle \text{HSP} \rangle = \text{HSP}_a + \text{HSP}_b$; the average HSP value is fixed: $\langle \text{HSP} \rangle = 0.47 \muM$; HSF1 is lognormally distributed with a 0.04 \muM average; and the three distributions (HSP$_a$, HSP$_b$, and HSF1) have the same width ($\sigma = 0.3$). Two uncorrelated isoforms, with a similar abundance, narrow the total HSP distribution (Fig. 10C, black lines) and quench the foci intensity distribution and thus the cell-to-cell variability (Fig. 10D). The foci intensity distribution quenching thus increases with the number of HSP isoforms having the same average and variance (Fig. 10E).

**Discussion**

This study aims to better understand the molecular origin of phenotypic heterogeneity that will be crucial to work around resistance of a subpopulation of cells upon cancer treatment. We also provide here a test for predictability of phenotypic heterogeneity by a mathematical model of the HSRN. We focus on HSRN because most anticancer treatments will activate this stress response. Moreover, HSRN is a good model system as it is well characterized in the literature and heat shock provides a homogeneous way to treat an entire cell population.

We combine high-throughput single-cell fluorescence experiments and mathematical modeling of the genetic regulation network. We first highlight an unexpected wide cell-to-cell variability in the activation of HSRN. We show that variability of the heat stress response is
largely the result of cell-to-cell basal HSP-level heterogeneity. Surprisingly, only a narrow variation in HSP basal level ($r \approx 0.3$) is sufficient to induce the observed cell-to-cell variability. In the model, heterogeneity amplification is induced by the sequestration mechanism at the core of HSRN activation. Immuno-fluorescence labeling experiments confirm that such an HSP expression level distribution explains a significant fraction of the heterogeneity and that the response amplitude depends on HSP expression level via the predicted mathematical relationship. We also show how the joint measurement of HSF1 and a chaperone determines the free fraction of chaperone in each cell. However, this determination is only possible when the nSBs are activated.

The response continuum observed in HSRN activation is a novel and surprising result that both completes the well-established biochemical data in the field and renews their interpretation. Sequestration cascades lead to hypersensitive response; heterogeneity amplification is thus achieved as the stimulus reaches the response hypersensitive threshold. At a fixed stimulus level, the initial state of the cellular proteome determines the HSRN cellular activation response. This response may be mainly classified in three phenotypic clusters: (a) no activation, (b) transient activation, or (c) sustained activation. In a cell population, all cells have a different proteome, and thus, all three types of activation dynamics are found for a given stress stimulus. We found that the probability of sustained activation increases with the stimulus level. Therefore, the averaged dynamical response measured by biochemical measurements (averages over a cell population) characterizes more the occurrence of the various phenotypes than the dynamics associated with a specific stress stimulus. Furthermore, if the sequestration cascades create a heterogeneity amplifier in the case of chronic stress ($T \geq 43 \, ^\circ\mathrm{C}$), they also create a heterogeneity amplifier.
collapse in the case of a mild stress. With this viewpoint, hypersensitivity of the stimuli-to-response curve could be a strategy to quench the protein expression heterogeneity below a given stimulus threshold.

We show that variation in HSP72 and HSC70 molecular chaperones plays a major role in CCRV. This is expected within the framework of titration model for HSF1 as HSP70 family that is shown to interact strongly with HSF1 transcription factor in unstressed cells [41]. This interaction was shown to be responsible for HSF1 sequestration in the absence of stress and desequestration from HSP70 that is crucial for HSRN activation in yeast [45]. We have tested whether CCRV could also arise from level variations in HSP90, another important chaperone. While HSP90 exhibits weakly interaction with HSF1 without stress [46], recent results suggest that sequestration may not be the important role of HSP90 in HSF1 regulation [47]. Instead, HSP90 interacts with transcriptionally active HSF1 trimers [48] and newly synthesized HSP90 may regulate HSF1 by attenuating its ability to promote transcription when bound to HSE in DNA [47]. Interestingly, at the single-cell level, we do not find correlation between HSRN activation and HSP90 copy number (data not shown). These results are consistent with the fact that our readout (nSBs) is a measurement of HSRN activation but does not reflect transcriptional activity per se.

Human HSP70 expression was shown to vary with the cell cycle stage [49]. However, in our experimental conditions, we do not find a significant correlation between single-cell DNA content (assessed by Hoechst fluorescence level) and HSP72 or HSC70 nuclear concentration. Instead, HSP expression level distribution could be attributed to transcriptional bursts intrinsically amplified by mRNA processing that causes substantial noise amplification at the protein level [50]. Recent experiments in yeast have revealed that HSF1 hyperphosphorylation is another source of variability in HSRN [51]. Such post-translational modifications control HSF1 activity on HSE rather than its activation in the cytosol [45]. HSF1 phosphorylation could therefore play a role in the CCRV we observe, even if we focus on activation of HSF1, because it might induce variations in HSP72 transcription rate upon stress. In fact, HSP72 expression level only slightly increases in 1 h upon stress (Fig. 7) without significant variation in its span (Fig. 8). Moreover, the effect of HSC70 (which is not stress-induced) on CCRV confirms the existence of a variability source, distinct from HSF1 phosphorylation, where the prestimulus cellular state at least partially determines single-cell stress response.

HSP72 and HSC70 play a similar role in the refolding of misfolded proteins, and their expression levels are not correlated. From the network point of view, this redundancy is intriguing and the only functional role we identify is an ability to quench CCRV over the cell population. However, we do not state that this is the principal explanation of HSP redundancy as long as the CCRV harmfulness is not clearly established.

Our results highlight that the sequestration cascade mechanisms leading to the titration of HSF1 by basal HSP and MFP can control with ultrasensitivity the stress response. It is a sufficient guideline of a regulation network that describes the cellular heat shock response at both the population and the single-cell levels. In this latter case, the HSP stochastic expression variability explains the observed phenotypic heterogeneity. Therefore, the ability to control the HSPs expression distribution, and not only its averaged expression level, should imply the ability to better...
control the phenotypic heterogeneity and then to potentially reduce a therapy resistant subset of cells. Hypersensitivity of HSRN is a feature shared by several stress-induced biological networks. As the amplification of heterogeneity is due more to hypersensitivity of the response than to the molecular mechanisms that engender it (sequestration in our case), the results and methods developed here could therefore be extended to other networks of stress and more broadly to hypersensitive networks.

Materials and methods

Cell culture and cell transfection

The HeLa human cervical cancer cell line (CCL-2™) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These adherent cells were grown as monolayer in Dulbecco’s modified Eagle’s medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% (v/v) FBS (Life Technologies, Saint-Aubin, France), 1% L-glutamine (2 mm), and 1% (v/v) penicillin/streptomycin (100 IU·mL⁻¹) (Lonza). Cell cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ (v/v) and passaged at confluence (twice a week) using 0.05% trypsin/0.53 mM ethylenediaminetetraacetate (EDTA; Lonza). HeLa growing cells were routinely screened for the presence of mycoplasma using DNA staining with the nuclear dye Hoechst 33342 (1:10 000 dilution) (Sigma-Aldrich, L’Isle d’Abeau Chesnes, France) to avoid collecting data from unknowingly contaminated cell cultures.

Wild-type HeLa cells (HeLa WT) were transfected with a plasmid expressing the human full-length HSF1 fused to eGFP. The plasmid was kindly provided by C. Vourc’h (Université Joseph-Fourier, Grenoble, France) and built as previously described [52]. Briefly, PCR amplification allowed to obtain the coding sequence for human HSF1 that was cloned into pcDNA N3 vector (Clontech Laboratories, Mountain View, CA, USA); the plasmid was then verified by sequencing (GATC Biotech, Constance, Germany). The transfection (of wild-type HeLa cells with the HSF1:eGFP plasmid) was carried out using FuGENE® HD transfection reagent (Promega, Charbonnières, France) according to the manufacturer’s instructions. The stable HSF1:eGFP-transfected (HeLa HSF1:eGFP) cell line was then established under selective pressure by 1000 μg·mL⁻¹ geneticin (Life Technologies) followed by selection of a single GFP-positive cell by flow cell sorting system (FACSAria III; Becton Dickinson, San Jose, CA, USA).

All experiments were performed on 2-day-old cell cultures (50% confluence) prepared by seeding 1.8 × 10⁶ cells into 35-mm dishes (Sarstedt, Marnay, France) in complete DMEM without phenol red.

Immunofluorescence staining of HSPs and HSF1

After 48 h of culture, HeLa WT and HeLa HSF1:eGFP cells were heated at 43 °C for 1 h in our homemade incubator controlled in temperature and gas conditions [53]. At the end of the thermal stress, and in parallel with the unstressed samples (control, 37 °C), cells were immediately rinsed with Dulbecco’s phosphate-buffered saline (DPBS; Lonza) and fixed in 4% paraformaldehyde in DPBS for 10–15 min at room temperature (RT). After washing three times with DPBS, samples were incubated for 30 min at RT in DPBS containing 0.3% Triton X-100 and 5% goat serum (v/v) allowing permeabilization of cells and blocking of nonspecific binding sites. Cells were then incubated overnight at 4 °C with monoclonal primary antibody as follows: mouse anti-HSC70 (1:100 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), or mouse anti-HSP72 (1:100 dilution; Enzo Life Sciences, Villeurbanne, France), or rabbit anti-HSF1 antibody (Enzo Life Sciences). Subsequently, samples were washed and incubated for 90 min at RT with either a goat anti-mouse (for HSC70/HSP72 expression) or a goat anti-rabbit (for HSF1 expression) secondary antibody conjugated to Alexa Fluor-594 (Life Technologies). A DNA staining using Hoechst 33342 (1:10 000) was also performed for all samples, to allow the automatic detection of nuclear areas for image analysis.

Live-/fixed-cell imaging

HeLa cells were cultured in 35-mm dishes (Sarstedt) at approximately 50% confluence. Samples were placed on a Nikon Ti-E microscope with motorized filters wheel equipped with a XY-motorized stage (ASI, Eugene, OR, USA). Cells were imaged through a 40x objective (NA = 0.6; Nikon) on a sCMOS camera (Orca-Flash LT, Hamamatsu, Shizuoka, Japan). We set the camera binning to 2 resulting in an effective pixel size of 325 nm. Illumination for fluorescence and brightfield imaging were achieved through custom-built optical system (components from Thorlabs). We used LED light source (Thorlabs, Newton, NJ, USA) for synchronization of illumination with other apparatus. Exposure time was set to 150 ms for all experiments and for each fluorescence channel as well as brightfield illumination. Light power density, filter set, and LED for each type of experiments are summarized in Table 1. We use a custom-built acquisition software written in LABVIEW (Austin, TX, USA) to control the setup.

For live-cell experiments, culture dishes were placed in a custom-built incubator that regulates temperature, humidity, and atmosphere. The incubator was described in Ref. [53]. Cells were maintained at 37 °C for 1 h and heat-shocked at 41 °C, 42 °C, or 43 °C for 3 h by increasing the incubator temperature. Time evolution of the nuclear stress body foci was monitored in real time. In order to increase the output rate of the experiment, we acquired data for 10 different
fields of view in the same dish (by the use of the motorized stage) leading to the tracking of approximately 200 cells per experiment. Two consecutive fields of view were separated by approximately 300 µm. To account for focusing drift and to allow image segmentation, we acquired for each field of view a z-stack of nine images per channel by moving the objective lens along the optical axis. Two consecutive images of the stack were defocused by approximately 2 µm. We acquired z-stack at a rate of 0.5 image min⁻¹.

For fixed-cell imaging, heat shocks were performed in the same incubator as the live-cell data. Cells were shocked for 1 h at 43 °C and then fixed right after. After fixation and immunolabeling (protocols described below), the cells were imaged on the same microscope. For these experiments, we used the Nikon Perfect Focus System and thus did not acquire z-stack. We imaged 400 positions per condition leading to approximately 8000 cells per experiment.

**Image processing and analysis**

All image processing and data analysis were performed using custom-written algorithms either in FORTRAN or in MATLAB (Natick, MA, USA).

For time-lapse microscopy experiments, we first estimated best focus for each z-stack by the use of a contrast function [54]. Best focus was estimated from fluorescence images. We did not find significant defocusing between fluorescence and brightfield in our experimental conditions. Cells were automatically segmented using brightfield image z-stack. For this, we took advantage of the fact that gray level varies across the z-stack for pixels located in cells, while such gray level was approximately constant for background pixels and pixels at the periphery of the cells. Image segmentation was visually inspected after image processing. Corrections to cell segmentation were carried out when necessary via a custom-written semi-automated graphical interface by either removing false positives or correcting masks. After cell segmentation, the cells were tracked by simply linking the closest cell found in the next image. Visual inspection of the tracking did not reveal errors as the cells do not move significantly in the interval between two acquisitions. We then estimated background for fluorescence images by convolving the raw data with a 30-pixel-wide Gaussian kernel (larger than cell size) and averaging across the z-stack. Background was subtracted to raw data for further analysis. The total HSF1:eGFP intensity was simply estimated by integrating fluorescence intensity over the whole-cell mask. HSF1:eGFP foci were automatically detected by the use of wavelet transform with a wavelet radius of 2 pixels [55]. Only spots with maximum intensity higher than mean cell intensity were considered for further analysis. The F factor was defined as the integrated intensity found in all foci divided by the total cellular fluorescence.

For fixed-cell immunofluorescence experiments, image segmentation was achieved on images from HSP fluorescence channel for whole-cell segmentation and on the images from Hoechst fluorescence channel for the nucleus segmentation. We acquired fluorescence images of dishes filled with fluorescent dye for flat field correction. The dyes were coumarin for Hoechst channel, rhodamine 110 for GFP and Alexa Fluor 488 channel, and rhodamine B for Alexa Fluor 594 channel. After flat field correction, images were segmented using a modified Otsu thresholding method [56]. A constant background was subtracted before further analysis. F was defined as above, and HSP concentration was defined as the total fluorescence inside the nucleus divided by the nuclear area in arbitrary units. False-positive detections were removed by selecting a polygon in the Hoechst intensity versus nucleus area plane.

**HSF1, HSP72, and HSC70 are lognormally distributed**

HSF1, HSP72, and HSC70 concentrations were estimated in individual cells from single immunolabeling in both HeLa wild-type and in HeLa HSF1:eGFP cell lines. Experimental data were compared for two thermal conditions: without heat shock and 1 h after exposure to a temperature step-up from 37 °C to 43 °C. All three protein concentrations at both 37 °C and 43 °C were well fitted by a lognormal distribution: A random variable X follows a lognormal distribution of parameters (μ, σ) if its probability density function reads

\[
\frac{1}{X\sigma\sqrt{2\pi}} \exp\left(-\frac{1}{2} \left(\frac{\log(X) - \mu}{\sigma}\right)^2\right).
\]

**Mathematical model for HSRN**

The heat stress cellular response dynamic is mainly regulated by two complexes that both involve the chaperone proteins HSPs [27]. HSPs titrate the misfolded proteins, on the one hand, and their own transcription factor (HSF1) on the other. A reduced model of the cellular response to heat stress was constructed from a detailed kinetic one of the literature [27] under the following assumptions: (a) All protein species have a similar half-life; and (b) the assembly dynamics and assemblies of the protein complexes are adiabatically eliminated and equilibrium equations at the fixed points are approximated by rational functions [57].

In addition to the model developed in Ref. [27], the present model improves the regulation of the translation process via HSPs. In fact, heat shock proteins are requested to initiate the translation process; therefore, the sequestration of heat shock protein by misfolded protein reduces the ability of HSP to initiate the translation. Straightforwardly, we included in the modeling an HSP-dependent translation rate, which decreases as the free monomeric HSP form
vanishes. This mechanistic detail is crucial to describe the slow increase in foci dynamics during a 43 °C heat stress.

The equations of the model are as follows:

\[
\tau_0 \frac{d}{dt} \theta = 0_c - \theta, \tag{3a}
\]

\[
\tau_{\text{MFP}} \frac{d}{dt} [\text{MFP}] = \kappa(0) - \frac{[\text{MFP}]^2}{[\text{HSP}]+[\text{MFP}]} - k_r \frac{[\text{MFP}][\text{HSP}]}{[\text{HSP}]+[\text{MFP}]}, \tag{3b}
\]

\[
\tau_{\text{HSP}} \frac{d}{dt} [\text{HSP}] = \beta \frac{[\text{HSP}]}{H_0+[\text{HSP}]}[\text{mHSP}] - [\text{HSP}], \tag{3c}
\]

\[
\tau_{\text{mHSP}} \frac{d}{dt} [\text{mHSP}] = \mu + \lambda \frac{([\text{HSP}])_{\text{free}}^3}{S_0 + ([\text{HSP}])_{\text{free}}^3} - [\text{mHSP}], \tag{3d}
\]

\[
[\text{HSF1}]_{\text{free}} = \frac{[\text{HSF1}]_{\text{tot}}}{[\text{HSF1}]_{\text{tot}} + [\text{HSP}]}, \tag{3e}
\]

where \( t \) is time; \( \theta \) is the temperature of the cell environment measured in °C; [MFP] is the misfolded protein concentration; [mHSP] is the concentration of mRNA coding for HSP; [HSP] is the heat shock protein concentration; [HSF1]_{tot} is the heat shock factor 1 protein total concentration; and [HSF1]_{free} is the concentration of free HSF1 proteins (not bounded to HSP). The denaturation rate \( \kappa(0) \) is here the only temperature input. Mathematical expression of \( \kappa(0) \) was discussed in Ref. [58], and it takes the following form in the range 37–45 °C:

\[
\kappa(0) = k_d (1 - 0.4e^{37-0}) 1.4^{0-37}. \tag{4}
\]

In this framework, the fraction, named \( F_{\text{Th}} \), of HSF1 bound to nSBs at a given date \( t \) corresponds to the fraction of free HSF1 at power 3 because only trimers of HSF1 are bounded: \( F_{\text{Th}} = ([\text{HSF1}]_{\text{free}}/[\text{HSF1}]_{\text{tot}})^3 \) Given Eqn (3e), \( F_{\text{Th}} \) reads:

\[
F_{\text{Th}} = \left( \frac{1}{1 + \frac{[\text{HSF1}]_{\text{tot}}}{[\text{HSP}]_{\text{tot}}+\overline{[\text{MFP}]}} \right)^3, \tag{5}
\]

where \([\text{HSF1}]_{\text{tot}}, [\text{HSP}], \) and \([\text{MFP}]\) refer to the total HSF1, HSP, and MFP concentrations. The expression for \( F_{\text{Th}} \) given in Eqn (5) depends on two concentration ratios, \([\text{MFP}]_{\text{tot}} / [\text{HSP}]_{\text{tot}} \) and \([\text{HSP}]_{\text{tot}} / [\text{MFP}]_{\text{tot}} \), that reflect the two competitive complex formations: The first ratio reflects the MFP titration by HSPs, while the second ratio accounts for HSF1 titration by HSPs.

The \( \tau_0 \) parameter value, the incubator rise time, was measured experimentally; the \([\text{HSF1}]_{\text{tot}}, \tau_{\text{MFP}}, \tau_{\text{mHSP}}, \) and \( \tau_{\text{HSP}} \) parameter values were settled according to Refs [27,57]; and the other parameter values were optimized to adjust at best the average foci dynamics (Fig. 5). The signification and values of model parameters are summarized in Table 2.

To highlight the regulated HSP transcription role, we also introduced an alternative model where HSP is a parameter and not a dynamical variable, by removing from Eqn (3) the active HSP transcription. The modified model equation reads

\[
\tau_0 \frac{d}{dt} \theta = 0_c - \theta, \tag{6a}
\]

### Table 1. Imaging conditions.

| Fluorophore   | Light source | Excitation filter | Dichroic mirror | Emission filter | Light intensity (W·cm⁻²) |
|---------------|--------------|-------------------|-----------------|----------------|--------------------------|
| GFP           | M490L4       | FF02-482/18       | FF495-Di03      | FF02-620/28    | – 1.8                    |
| Alexa Fluor 488 | M490L4  | FF02-482/18       | FF495-Di03      | FF02-620/28    | – 1.8                    |
| Alexa Fluor 594 | MCVHL5  | FF01-538/20       | FF01-600/37     | 0.63           |
| Hoechst       | M365LP       | FF01-360/2        | FF416-Di01      | FF02-460/80    | – 1.35                   |
| Brightfield   | MCVHL3       | –                 | FF495-Di03      | FF02-620/28    | – 2.25                   |

### Table 2. Estimated parameters of the heat shock response network.

| Parameter                  | Unit | Description                      | Value  |
|----------------------------|------|----------------------------------|--------|
| \( k_d \)                 | µM   | Denaturation rate                | 1.76   |
| \( k_r \)                 |      | Renaturation rate                | 17.7   |
| \( \mu \)                 | µM   | HSP basal transcription rate     | 1.47 × 10⁻³ |
| \( \lambda \)             | µM   | HSP active transcription rate    | 0.78   |
| \( S_0 \)                 | µM   | HSP transcription regulation     | 0.18   |
| \( \beta \)               | µM   | HSP translation rate             | 10     |
| \( H_0 \)                 |      | Translation regulation threshold | 0.32   |
| \([\text{HSF1}]_{\text{tot}}\) | µM   | HSF1 concentration              | 4.0 × 10⁻² |
| \( \tau_{\text{temp}} \)  | h    | Incubator rise time              | 1/15   |
| \( \tau_{\text{MFP}} \)   | h    | MFP lifetime                     | 0.5    |
| \( \tau_{\text{mHSP}} \)  | h    | mHSP lifetime                    | 1      |
| \( \tau_{\text{HSP}} \)   | h    | HSP lifetime                     | 10     |
\[
\tau_{MFP} \frac{d}{dt} \langle MFP \rangle = \kappa(\theta) - \frac{[MFP]^2}{[HSP] + [MFP]} - k_r \frac{[MFP][HSP]}{[HSP] + [MFP]}
\]

The values of parameters are identical to those summarized in Table 2. Figure 5 displays the numerical outputs of modified model Eqn (6) with \([HSP] = 0.479 \mu M\) (which is the HSP concentration at 37 °C obtained with Eqn 3). Model Eqn (6) correctly captures the first hour of the nSB dynamic but fails to describe the nSB relaxation because numerical outputs converge to fixed point values (Fig. 5, blue lines). The regulated HSP transcription is crucial to validate the used power index. As a reference, we mimicked fluorescence microscopy with expression 5, especially to quantitatively describe nSB relaxation.

In practice, we sought to compare measurements from fluorescence microscopy with expression 5, especially to validate the used power index. As a reference, we mimicked the effect of HSF1 and HSP lognormal distribution on the \(F_{Th}\) distribution. To do this in a simple setting, we computed the \(F_{Th}\) value 1 h after the stress onset from model Eqn (6) with a lognormal distribution of \([HSP]\) and \([HSF1]\) having the same averages as model Eqn (3), and the same width as that measured by immunofluorescence (Fig. 7). As the \(F_{Th}\) mathematical expression is highly non-linear, the obtained average value \(\langle F_{Th} \rangle\) did not match the \(F_{Th}\) value obtained with average value of HSP and HSF1. We thus optimized again \(k_d\) and \(k_r\) to fit \(\langle F_{Th} \rangle\) with \(\langle f \rangle\) 1 h after the stress onset for the three temperatures (41 °C, 42 °C, and 43 °C). We thus obtain \(k_r = 1.31 \mu M\) and \(k_d = 19.59\). All numerical calculations implying HSF1 and HSP lognormal distributions used the modified \(k_r\) and \(k_d\) values.

Acknowledgements

This work was partially supported by the LABEX CEMPI (ANR-11-LABX-0007), as well as by the Ministry of Higher Education and Research, Hauts de France Council, and European Regional Development Fund (ERDF) through the Contrat de Projets Etat-Region (CPER Photonics for Society P4S).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

MG, FA, AP, and QT performed the research; MG, FA, EC, and QT designed the research; MG, FA, and QT analyzed the data; and FA, EC, and QT wrote the paper.

References

1 LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, Fong S, Schwall R, Sinicropi D & Ashkenazi A (2002) Tumor-cell resistance to death receptor–induced apoptosis through mutational inactivation of the proapoptotic Bel-2 homolog Bax. Nat Med 8, 274.
2 Albeck JG, Burke JM, Aldridge BB, Zhang M, Lauffenburger DA & Sorger PK (2008) Quantitative analysis of pathways controlling extrinsic apoptosis in single cells. Mol Cell 30, 11–25.
3 Feinerman O, Veiga J, Dorfman JR, Germain RN & Altan-Bonnet G (2008) Variability and robustness in t cell activation from regulated heterogeneity in protein levels. Science 321, 1081–1084.
4 Gascoigne KE & Taylor SS (2008) Cancer cells display profound intra-and interline variation following prolonged exposure to antimitotic drugs. Cancer Cell 14, 111–122.
5 Orth JD, Tang Y, Shi J, Loy CT, Amendt C, Wilm C, Zenke FT & Mitchison TJ (2008) Quantitative live imaging of cancer and normal cells treated with kinesin-5 inhibitors indicates significant differences in phenotypic responses and cell fate. Mol Cancer Ther 7, 3480–3489.
6 Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud Ø, Gjertsen BT & Nolan GP (2004) Single cell profiling of potentiated phospho-protein networks in cancer cells. Cell 118, 217–228.
7 Cohen AA, Geva-Zatorsky N, Eden E, Frenkel-Morgenstern M, Issaeva I, Sigal A, Milo R, Cohen-Saidon C, Liron Y, Kam Z et al. (2008) Dynamic proteomics of individual cancer cells in response to a drug. Science 322, 1511–1516.
8 Geva-Zatorsky N, Rosenfeld N, Itzkovitz S, Milo R, Sigal A, Dekel E, Yarnitzky T, Liron Y, Polak P, Lahav G et al. (2006) Oscillations and variability in the p53 system. Mol Syst Biol 2, 2006.0033. https://doi.org/10.1038/msb4100068
9 Niepel M, Spencer SL & Sorger PK (2009) Non-genetic cell-to-cell variability and the consequences for pharmacology. Curr Opin Chem Biol 13, 556–561.
10 Almendro V, Marusyk A & Polyak K (2013) Cellular heterogeneity and molecular evolution in cancer. Annu Rev Pathol 8, 277–302.
11 Kaern M, Elston TC, Blake WJ & Collins JJ (2005) Stochasticity in gene expression: from theories to phenotypes. Nat Rev Genet 6, 451.
12 Sigal A, Milo R, Cohen A, Geva-Zatorsky N, Klein Y, Liron Y, Rosenfeld N, Danon T, Perzov N & Alon U (2006) Variability and memory of protein levels in human cells. Nature 444, 643.
13 Huang S (2009) Non-genetic heterogeneity of cells in development: more than just noise. Development 136, 3853–3862.
Protein level variability determines phenotypic heterogeneity

M. Guilbert et al.

14 Acar M, Mettetal JT & Van Oudenaarden A (2008) Stochastic switching as a survival strategy in fluctuating environments. Nat Genet 40, 471–475.
15 Pelet B & Thommen Q (2016) Adaptive benefits of storage strategy and dual AMPK/TOR signaling in metabolic stress response. PLoS ONE 11, e0160247.
16 Spencer SL, Gaudet S, Albeck JG, Burke JM & Sorger PK (2009) Non-genetic origins of cell-to-cell variability in trail-induced apoptosis. Nature 459, 428–432.
17 Flusberg DA & Sorger PK (2015) Surviving apoptosis: life-death signaling in single cells. Trends Cell Biol 25, 446–458.
18 Roux J, Hafner M, Bandara S, Sims JJ, Hudson H, Chai D & Sorger PK (2015) Fractional killing arises from cell-to-cell variability in overcoming a caspase activity threshold. Mol Syst Biol 11, 803.
19 Bertaux F, Stoma S, Drasdo D & Batt G (2014) Modeling dynamics of cell-to-cell variability in trail-induced apoptosis explains fractional killing and predicts reversible resistance. PLoS Comput Biol 10, e1003893.
20 Behar M, Barken D, Werner SL & Hoffmann A (2013) The dynamics of signaling as a pharmacological target. Cell 155, 448–461.
21 Reyes J & Lahav G (2018) Leveraging and coping with uncertainty in the response of individual cells to therapy. Curr Opin Biotechnol 51, 109–115.
22 Loewer A & Lahav G (2011) We are all individuals: causes and consequences of non-genetic heterogeneity in mammalian cells. Curr Opin Genet Dev 21, 753–758.
23 Altschuler SJ & Wu LF (2010) Cellular heterogeneity: do differences make a difference? Cell 141, 559–563.
24 Goldbeter A & Koshland D (1984) Ultrainsensitivity in biochemical systems controlled by covalent modification. Interplay between zero-order and multistep effects. J Biol Chem 259, 14441–14447.
25 Buchler NE & Cross FR (2009) Protein sequestration generates a flexible ultrasensitive response in a genetic network. Mol Syst Biol 5, 272.
26 Morimoto RI (2012) The heat shock response: systems biology of proteotoxic stress in aging and disease. Cold Spring Harb Symp Quant Biol 76, 91–99.
27 Sivény A, Courtade E & Thommen Q (2016) A minimal titration model of the mammalian dynamical heat shock response. Phys Biol 13, 066008.
28 Jolly C & Morimoto RI (2000) Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst 92, 1564–1572.
29 Biamonti G & Vourc’h C (2010) Nuclear stress bodies. Cold Spring Harb Perspect Biol 2, a000695.
30 Abravaya K, Phillips B & Morimoto R (1991) Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures. Genes Dev 5, 2117–2127.
31 Kline M & Morimoto R (1997) Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. Mol Cell Biol 17, 2107–2115.
32 Sarge KD, Murphy SP & Morimoto RI (1993) Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol Cell Biol 13, 1392–1407.
33 Cotto J, Kline M & Morimoto R (1996) Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. J Biol Chem 271, 3355–3358.
34 Mosser D, Theodorakis N & Morimoto R (1988) Coordinate changes in heat shock element-binding activity and hsp70 gene transcription rates in human cells. Mol Cell Biol 8, 4736–4744.
35 Baler R, Dahl G & Voellmy R (1993) Activation of human heat shock genes is accompanied by oligomerization, modification, and rapid translocation of heat shock transcription factor HSFl. Mol Cell Biol 13, 2486–2496.
36 Holmberg C, Tran S, Eriksson J & Sistonen L (2002) Multisite phosphorylation provides sophisticated regulation of transcription factors. Trends Biochem Sci 27, 619–627.
37 Cotto J, Fox S & Morimoto R (1997) HSFl granules: a novel stress-induced nuclear compartment of human cells. J Cell Sci 110, 2925–2934.
38 Boulon S, Westman B, Hutten S, Boisvert F & Lamond A (2010) The nucleolus under stress. Mol Cell 40, 216–227.
39 Neumüller RA & Knoblich JA (2009) Dividing cellular asymmetry: asymmetric cell division and its implications for stem cells and cancer. Genes Dev 23, 2675–2699.
40 Whitley D, Goldberg SP & Jordan WD (1999) Heat shock proteins: a review of the molecular chaperones. J Vasc Surg 29, 748–751.
41 Shi Y, Mosser D & Morimoto R (1998) Molecular chaperones as HSFl-specific transcriptional repressors. Genes Dev 12, 654–666.
42 Gehing M-J & Sambrook J (1992) Protein folding in the cell. Nature 355, 33–45.
43 Tavaria M, Gabriele T, Kola I & Anderson RL (1996) A hitchhiker’s guide to the human hsp70 family. Cell Stress Chaperones 1, 23–28.
44 Mercier PA, Winegarden NA & Westwood JT (1999) Human heat shock factor 1 is predominantly a nuclear protein before and after heat stress. J Cell Sci 112, 2765–2774.
45 Zheng X, Krakowiak J, Patel N, Beyzavi A, Ezike J, Khalil AS & Pincus D (2016) Dynamic control of HsFl during heat shock by a chaperone switch and phosphorylation. Elife 5, e18638.
46 Zou J, Guo Y, Guettouche T, Smith DF & Voellmy R (1998) Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. Cell 94, 471–480.

47 Kijima T, Prince TL, Tigue ML, Yim KH, Schwartz H, Beebe K, Lee S, Budzynski MA, Williams H, Trepel JB et al. (2018) Hsp90 inhibitors disrupt a transient HSP90-HSF1 interaction and identify a noncanonical model of HSP90-mediated HSF1 regulation. Sci Rep 8, 6976.

48 Conde R, Belak ZR, Nair M, O’Carroll RF & Ovsenek N (2009) Modulation of Hsf1 activity by novobiocin and geldanamycin. Biochem Cell Biol 87, 845–851.

49 Milarski KL & Morimoto RI (1986) Expression of human hsp70 during the synthetic phase of the cell cycle. Proc Natl Acad Sci USA 83, 9517–9521.

50 Hansen AS & Shea EKO (2016) Encoding four gene expression programs in the activation dynamics of a single transcription factor. Curr Biol 26, R269–R271.

51 Zheng X, Beyzavi A, Krakowiak J, Patel N, Khalil AS & Pincus D (2018) Hsf1 phosphorylation generates cell-to-cell variation in Hsp70 levels and promotes phenotypic plasticity. Cell Rep 22, 3099–3106.

52 Herbomel G, Kloster-Landsberg M, Folco EG, Col E, Usson Y, Vourc’h C, Delon A & Souchier C (2013) Dynamics of the full length and mutated heat shock factor 1 in human cells. PLoS ONE 8, e67566.

53 Anquez F, El Yazidi-Belkoura I, Randoux S, Suret P & Courtade E (2012) Cancerous cell death from sensitizer free photoactivation of singlet oxygen. Photochem Photobiol 88, 167–174.

54 Price JH & Gough DA (1994) Comparison of phase-contrast and fluorescence digital autofocus for scanning microscopy. Cytometry 16, 283–297.

55 Olivo-Marin J-C (2002) Extraction of spots in biological images using multiscale products. Pattern Recognit 35, 1989–1996.

56 Otsu N (1979) A threshold selection method from gray-level histograms. IEEE Trans Syst Man Cybern 9, 62–66.

57 Ladjimi M, Labavić D, Guilbert M, Anquez F, Pruvost A, Courtade E, Pfeuty B & Thommen Q (2019) Dynamical thermal dose models and dose time-profile effects. Int J Hyperth 36, 721–729.

58 Peper A, Grimbergen C, Spaan J, Souren J & Wijk R (1998) A mathematical model of the hsp70 regulation in the cell. Int J Hyperth 14, 97–124.