

On a fundamental structure of gene networks in living cells

Nataly Kravchenko-Balasha^{a,b}, Alexander Levitzki^a, Andrew Goldstein^c, Varda Rotter^d, A. Gross^b, F. Remacle^{b,e}, and R. D. Levine^{b,f,g,1}

^aUnit of Cellular Signaling, Department of Biological Chemistry, The Alexander Silberman Institute of Life Sciences and ^bThe Fritz Haber Research Center for Molecular Dynamics, Institute of Chemistry, Hebrew University of Jerusalem, Jerusalem 91904, Israel; ^cMolecular Biology Institute, ^dCrump Institute for Molecular Imaging, and ^eDepartment of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095; ^fDepartment of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel; and ^gDépartement de Chimie, B6c, Université de Liège, B-4000 Liège, Belgium

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Computers are organized into hardware and software. Using a theoretical approach to identify patterns in gene expression in a variety of species, organs, and cell types, we found that biological systems similarly are comprised of a relatively unchanging hardware-like gene pattern. Orthogonal patterns of software-like transcripts vary greatly, even among tumors of the same type from different individuals. Two distinguishable classes could be identified within the hardware-like component: those transcripts that are highly expressed and stable and an adaptable subset with lower expression that respond to external stimuli. Importantly, we demonstrate that this structure is conserved across organisms. Deletions of transcripts from the highly stable core are predicted to result in cell mortality. The approach provides a conceptual thermodynamic-like framework for the analysis of gene-expression levels and networks and their variations in diseased cells.

disease patterns | firmware | genomics | surprisal analysis | thermodynamic analysis

In biological studies, gene-expression networks are interrogated routinely to recognize transcripts and pathways that vary significantly upon experimental manipulation or among samples displaying distinct phenotypes. Identifying the significance of variation requires a definition and understanding of transcripts whose expression levels are steady and therefore can serve as a baseline from which the change is measured. We expect that the baseline includes the transcripts often referred to as “housekeeping genes,” the genes that maintain the basic cellular function. However, many familiar housekeeping genes vary significantly under different experimental conditions (1), complicating their utility as a baseline for gene-expression studies and calling into question our understanding of their cellular function. We use a thermodynamic-like theoretical approach to identify a set of steady-state genes in living cells. These genes define the stable baseline. Interestingly, two distinguishable groups could be resolved within this “steady” set of transcripts: genes that are highly expressed, stable, and robust and a flexible subset with lower expression levels that can vary depending on the experimental manipulations and are subject to epigenetic transformations. Importantly, we demonstrate that this structure is conserved across organisms.

The set of steady transcripts is reminiscent of what is called “firmware” in electronic systems and computing. Firmware enables the very basic functioning of a consumer electronic device.

The devices it enables can range in complexity from washing machines or remote controllers of TV sets to smart mobile phones. Engineers discern two classes that make up firmware: low-level firmware, which is part of the hardware of the device, and high-level firmware, the operational instructions that reside in the device. These two levels are analogous to the two classes, housekeeping and signaling, that we identify within the steady set of genes.

The analogy perhaps can be extended further, because it is possible, from the outside, to induce the high-level firmware to

reprogram itself and thereby provide additional applications or to express hitherto hidden functionalities of the device, such as the switch to glycolysis (2) in cancerous cells. Furthermore, landmark studies have demonstrated a corresponding and newly recognized plasticity in mammalian cells: Like firmware, gene networks in a differentiated cell can be induced by extrinsic influences, including transcription factors and small molecules, to alter their normal state, reprogram to an embryonic-like state, and perform hitherto hidden functions. On the other hand, changes in the housekeeping genes are expected to be lethal. This feature, too, is reminiscent of electronic devices.

To complement the set of transcripts that constitute the steady pattern, we characterize the transcription patterns underlying the process of oncogenic transformation and other changes in biological processes. The expression levels of the transcripts that belong to these patterns vary significantly in different types of cancers and among different patients who have the same type of cancer. The dramatic variations among patients who have the same disease have clear implications for translation and personalized medicine. At the same time, we show that these variations are minor compared with the level of expression of the stable transcripts. It is possible to extend the computer analogy and relate software to those easily modified and personalized transcription patterns that describe the biological change.

A pattern is a set of genes whose expression levels do not vary with time, as in the steady state, or that vary in concert (i.e., all the transcription levels are time-dependent in the same way).

The analogy that we make with computer architecture is complementary to the well-developed characterization of local motifs in transcription networks (3, 4). The aim in characterizing local motifs is to discern connections between a few genes that act like simple logic gates. Here, instead, we examine the flow of information in the whole genome to identify large groups of transcripts that act in concert. There are other important methods that analyze expression level changes in the cellular process (5). Many are local (i.e., bottom up), such as the Bayesian methods based on elucidating the relationships between a few genes at a time (6–11). The approaches based on information theory that rely on the concept of statistical entropy (8–10, 12) differ from the present work in that we use thermodynamics to define the physical entropy, the free energy of the transcripts, and thereby the steady state. Reverse-engineering algorithms based on chemical

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¹To whom correspondence should be addressed. E-mail: rafi@fh.huji.ac.il.

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generate a connected stable network, the degree and structure of the network of unstable transcripts is temporal and is somewhat dependent on stimuli and available partners.

Conservation of the Steady-State Pattern in Different Cells and Organisms. The distribution of the G_{i0} values among the cellular functional modules remains essentially the same in the steady-state pattern of the different cellular models, such as TGF- β -treated lung cancer cells (28), WI-38 transformed fibroblasts (27), HPV16 immortalized keratinocytes from four different stages of transformation (31), the renal carcinoma metastatic model (32), TGF- β -treated hematopoietic stem cells, and dendritic differentiated cells (33). The detailed analysis of the distribution among functional groups is provided in *SI Appendix, Text Section 2*.

The most stable transcripts (i.e., the core transcripts with the lowest values of G_{i0} discussed above) usually include ribosome, protein translation, and energy pathways modules (see, for example, *SI Appendix, Tables S3, S7, S10, and S11*). The transcripts with mid G_{i0} values participate in the cell cycle and DNA/RNA metabolism (*SI Appendix, Tables S8 and S12*). The adaptable, most easily modified, transcripts, those with the highest G_{i0} values, usually participate in signal transduction, morphogenesis, and cell communication modules (*SI Appendix, Tables S9 and S13*). It is important to note that the majority of the transcripts have mid G_{i0} values (see, for example, Fig. 1 and *SI Appendix, Fig. S3*).

Quantifying the Conservation of the Steady-State Pattern in Different Cells. A number of distinct experimental trajectories have been measured for the WI-38 cancer model. These trajectories include the development of normal cells (trajectory 134), immortalization (trajectory 156), and cancer development (trajectory 15781012) (see refs. 22 and 23 and *SI Appendix, Fig. S4* for more details). We make a quantitative comparison of the steady-state expression in different trajectories using the notion of dissimilarity discussed in *Experimental Procedures Box 3*. We make the comparisons by calculating the overlap of two different vectors: $\sum_i G_{i0}^{(\text{trj}X)} G_{i0}^{(\text{trj}Y)}$ for the trajectories X and Y (see *Experimental Procedures, Box 3* for details). We know that $\sum_i G_{i0}^{(\text{trj}X)} G_{i0}^{(\text{trj}Y)} = 1$, because, for a given trajectory X , the weights are normalized (see *Experimental Procedures, Box 2*). Therefore, if two different trajectories have similar distributions of expression levels, the value of the overlap $\sum_i G_{i0}^{(\text{trj}X)} G_{i0}^{(\text{trj}Y)}$ should be close to 1. For example, the scalar product of the G_0 vectors of two different trajectories in the cancer model system WI-38 is $\sum_i G_{i0}^{(156)} G_{i0}^{(15781012)} = 0.998$. (See *Experimental Procedures, Box 3* for further discussion of the notion of a scalar product.) Similar results are found for all scalar products among the five different trajectories in the WI-38 system. The steady-state core remains essentially unchanged despite the different pathways that have been characterized for the WI-38 cellular system.

We find that the concept of the steady-state core is equally applicable to other cellular systems. Using gene-expression data for different cell types, we calculated the G_0 vector of lung cancer cells (A549) treated with TGF- β to induce EMT (28); normal colon, primary carcinoma, and metastatic cells from two different patients (34); normal renal, tumor, and metastatic cells from three patients (32); prostate tissues taken from radical prostatectomy surgeries from four different patients and separated into highly purified populations of benign basal epithelial cells, benign luminal epithelial cells, and cancerous luminal cells (*SI Appendix, Text*); and human papilloma virus (HPV16) immortalized keratinocytes from four distinct time points in the route of transformation (31). We calculated the scalar product of the G_0 vectors of different cell types. Typical results are

$$\begin{aligned} \sum_i G_{i0}^{(\text{trj}156)} G_{i0}^{(\text{TGF}\beta \text{ cells})} &= 0.97, \quad \sum_i G_{i0}^{(\text{HF1 cells})} G_{i0}^{(\text{TGF}\beta \text{ cells})} = 0.96, \\ \sum_i G_{i0}^{(\text{colon cancer})} G_{i0}^{(\text{TGF}\beta \text{ cells})} &= 0.98, \\ \sum_i G_{i0}^{(\text{prostate cancer})} G_{i0}^{(\text{TGF}\beta \text{ cells})} &= 0.97. \end{aligned}$$

Because highly expressed transcripts have larger absolute G_{i0} values and therefore contribute most to the scalar products, the above results show that highly expressed stable transcripts are neither extensively suppressed nor amplified across different biological processes in human cells. Next we analyzed data (30) for skin tumor progression from three different disease stages (normal, benign papillomas, and malignant carcinomas) in 31 mice. The resulting 31 vectors were remarkably similar, with a scalar product > 0.993 for all mouse pairs (*SI Appendix, Fig. S5*). The distribution of the transcripts among functional groups in the steady-state pattern is similar to the distribution in human models. We further examined the steady-state pattern of phenelzine-treated *S. cerevisiae* grown for 200 min (29) and, as an extreme example, *Escherichia coli* grown on biofilms for 24 h (35); results are reported in *SI Appendix, Text Section 2*.

Comparing Disease-Induced Transcription Patterns of Different Patients. In contrast to the conservation of the steady state, we note that the transcription patterns that characterize the process of transformation vary significantly among different patients who have the same type of cancer. For example, the largest overlap for the first disease pattern in two different patients with renal metastatic cancer is 0.43, (*SI Appendix, Table S4*). The same result was obtained for two patients with colon carcinoma (ref. 34 and *SI Appendix, Table S5*) and for four patients with prostate cancer (*SI Appendix, Table S6*). The analysis of the key signaling proteins present in the pattern disease in colon and renal cancer samples, such as PTEN (a negative regulator of Akt pathway), different MAPK proteins, SRC, MYC, EGFR, PDGFR, IGFR, and NF κ BIL2 (a negative regulator of NF κ B) shows that these hub proteins have different, sometimes opposite, contributions in different patients who have the same type of cancer (*SI Appendix, Tables S44 and S54*).

As in human patients, the G_1 ($\alpha = 1$) arrays of the major transcription patterns for different mice were rather heterogeneous and very much uncorrelated (*SI Appendix, Fig. S5*). These results provide a potential explanation for the heterogeneity in patient tumor response to pathway inhibitors observed in clinical trials.

Stable Transcripts of the Steady-State Participate Less in the Process of Transformation. Transcripts that are significantly up- or down-regulated in the process of transformation are necessarily located at the edges of histogram describing the major transcription pattern (Fig. 1). Examination of the free energy values of these transcripts at the steady state revealed that the majority of these transcripts are located on the upper edge of the steady-state free energy histogram ($G_{i0\lambda_0}$; Fig. 1); that is, they are categorized among the less stable of the stable core transcripts. Quantitative data bearing on this point are discussed in *SI Appendix, Text, Sections 5 and 6* and Fig. S6. These transcripts belong to signal transduction and cell communication proteins and cell-cycle networks (*SI Appendix, Text, Section 6* and Fig. S6). Furthermore, the above analysis showed that the steady-state core transcripts with the lowest free energy are hardly influenced by any disease process (*SI Appendix, Text, Section 6* and Fig. S6).

From Steady-State Transcription Levels to the Functional Network. To examine the structure of the transcripts with the greatest

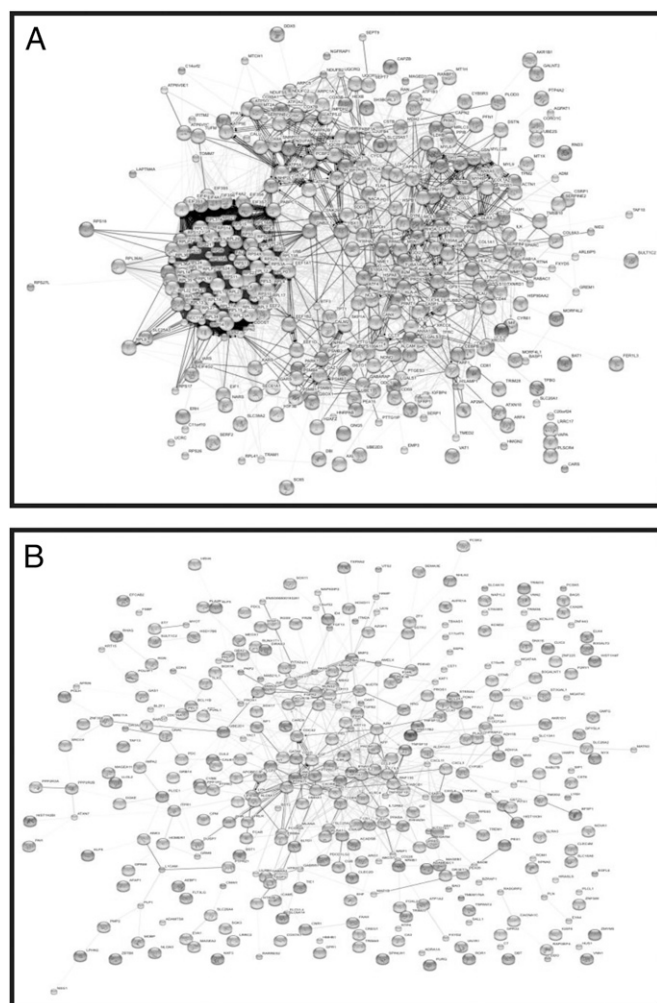


Fig. 2. Examination of the experimentally determined networks obtained from the G_{i0} values in the trajectory 15781012. (A) The 66 transcripts with the lowest (i.e., most stable) G_{i0} values (less than -0.018 according to *SI Appendix, Fig. S3*). (B) The 354 transcripts with the highest G_{i0} values (greater than -0.008 according to *SI Appendix, Fig. S3*). The validation of the high connectivity of the stable transcripts is shown in Fig. 3.

contribution to the steady state, we used the STRING software (36) that builds functional protein-association networks based on compiled available experimental evidence. As an example, consider the most stable transcripts, namely those with the lowest G_{i0} values (less than -0.018) in *SI Appendix, Fig. S3*. Fig. 2 shows that these transcripts generate a highly connected, experimentally validated network. The lowest G_{i0} values therefore are able to predict experimentally previously known protein-protein interactions. The least stable transcripts, those with the highest negative G_{i0} values (greater than -0.008) in *SI Appendix, Fig. S3* are much less connected than the transcripts with the lowest free energy (Fig. 2B). In the *SI Appendix* we show that the transcripts with either low or high G_{i1} values produce significantly less-connected experimentally validated networks (*SI Appendix, Fig. S7*). In *SI Appendix, Text Sections 1 and 3*, we discuss why the lowest G_{i0} values predict connectivity. We conclude that transcripts with stable gene expressions are more connected.

Free Energy Predicts the Functional Connectivity of Transcripts. To validate further the prediction of functional connectivity by free energy values, we built a heatmap according to the symmetric matrix, whose ij element is $G_{i0}G_{j0}$. Results for the process shown

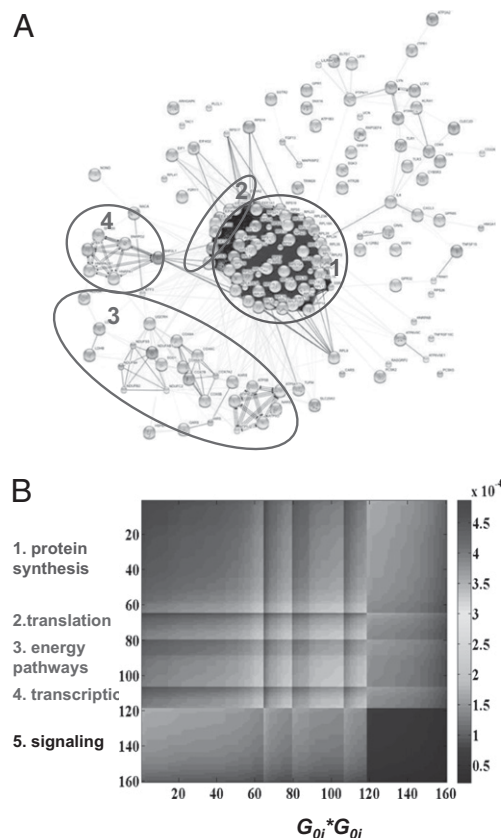


Fig. 3. Predicting the connectivity of transcripts in the trajectory 15781012. (A) Connectivity of 160 transcripts in the process of Fig. 1 according to the STRING software. The 120 most stable and the 40 least stable transcripts are shown. (B) A heatmap of the corresponding $G_{i0}G_{j0}$ values. The transcripts are labeled according to Gene Ontology categories. The transcripts with high G_{i0} values, group 5, are sparsely connected.

in Fig. 1 are compared in Fig. 3 with the known protein-protein interactions according to STRING. The values of the product $G_{i0}G_{j0}$ for 160 transcripts are represented in Fig. 3 by a heatmap (Fig. 3B). The most connected group in the heatmap comprises the transcripts with the lowest G_{i0} values. Transcripts in this group are involved primarily in protein synthesis. Transcripts involved in energy pathways and transcription are somewhat less connected. The transcripts with high free energy are hardly connected. The steady-state connectivity agrees well with the known interaction maps according to STRING (Fig. 3A).

Corresponding results for the TGF- β -treated A549 cells are shown in *SI Appendix, Fig. S8*. The interactions predicted by the heatmap also reflect known interactions according to STRING. The same result is found in other human cells.

Connectivity Networks Across Organisms. Finally, we argue that in other organisms, also, low G_{i0} values predict connectivity. In mice, for example we examined the process of carcinoma development (30) that included three stages: normal, benign, and carcinoma. The analysis showed that the steady-state connectivity that we calculated (Fig. 4B), agrees well with the known interaction maps according to STRING (Fig. 4A). The most connected group in the heatmap primarily comprises transcripts involved in protein synthesis. Transcripts involved in energy pathways are less connected. The transcripts with high free energy are hardly connected. Examination of data for other organisms showed similar results (*SI Appendix, Figs. S9 and S10*).

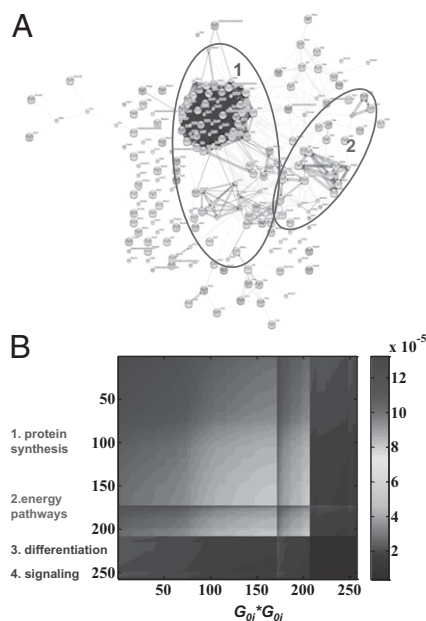


Fig. 4. Predicting the connectivity of transcripts as determined for carcinoma development in mice. (A) Connectivity of 250 transcripts (the 210 most stable and the 40 least stable transcripts) is shown. (B) A heatmap of the corresponding $G_{0i}G_{0j}$ values. The transcripts are labeled according to Gene Ontology categories. The transcripts with high G_{0i} values, groups 3 and 4, are sparsely connected.

Discussion

Using the measured distribution of expression levels of transcripts at several time points of a biological progression (*Experimental Procedures*), we determined the steady-state pattern and the transcription patterns that represent the deviations from the steady state caused by the ongoing process. The transcripts that contribute significantly to the steady state have much lower free energy values than the values of energy that can be produced by the particular process. The lower free energy explains why the steady state is robust.

The steady-state core network is conserved among different biological processes and across organisms. This result is consistent with previous studies showing that essential genes display lower variability (37, 38). We expect that the conservation of the core transcripts is essential for survival. On the other hand, we find that the transcription patterns that deviate from the steady state can vary significantly in the different types of cancer or even among different patients with the same type of cancer. Furthermore, we have found a remarkable convergence between our predicted structure of a steady-state network and the experimentally validated protein-interaction network in all the biological systems that we examined.

Functional analysis of the steady-state transcripts establishes that the protein network (which includes ribosomal RPS proteins, RPL proteins, and eukaryotic initiation and elongation factors) and energy metabolism networks (such as the oxidation phosphorylation chain network, including NDUF, COX transcripts, and ATP synthases) are the main contributors to the steady-state core set. Signal transduction and cell communication pathways that do not contribute significantly to the steady state and whose level is lower can vary in response to perturbations and are better able to respond to changing external stimuli. This set potentially would contain a larger number of therapeutic targets than the more stable core sequences.

In conclusion, surprisal analysis is able to predict the functionally connected stable steady-state network of a biological process by determining the free energy values of the mRNA

molecules. The steady-state pattern remains largely unchanged in different biological processes and across organisms and provides a baseline for measuring deviations caused by a perturbation or a disease. The deviations are software-like gene patterns that are uncorrelated with the steady state and that vary even among different individuals with tumors of the same type.

Experimental Procedures

Surprisal analysis identifies the steady state and also the transcription patterns that represent the biological change. Each transcription pattern is an enumeration of the participating transcripts. Surprisal analysis shows that all the transcripts belonging to the same pattern act in concert, contributing in the same manner over time. Each transcript contributes to a transcription pattern with its characteristic, time-independent weight. In Box 1, $G_{i\alpha}$ is the weight of transcript labeled i to the transcription pattern α . We use $\alpha = 0$ to designate the steady state; $\alpha = 1, 2, \dots$ are the transcription patterns of the disease. A key practical point is that very few (two or three) transcription patterns suffice to represent accurately the changes in the expression levels of transcripts caused by the biological process. We use the number $\lambda_{i\alpha}(t)$ as the value of the contribution of transcription pattern α at the time t . Surprisal analysis shows that very few $\lambda_{i\alpha}(t)$ values are not zero, so only a few transcription patterns contribute (22, 23). Surprisal analysis also identifies the steady state as the pattern whose value, λ_{i0} , does not vary with time.

Box 1. Surprisal Analysis Defines the Steady State.

$$\underbrace{\ln X_i(t)}_{\text{measured expression level of transcript } i \text{ at time point } t} = \underbrace{\ln X_i^0}_{\text{expression level of transcript } i \text{ at the steady state}} - \underbrace{\sum_{\alpha=1} G_{i\alpha} \lambda_{i\alpha}(t)}_{\text{deviation from the steady state due to the biological process}}$$

$\ln X_i^0 = -G_{i0}\lambda_0$ the fold level of transcript i at the steady state $\alpha = 0$. λ_0 does not depend on time. In refs. 22 and 23 we provide full details on how the steady state and the values of $\lambda_{i\alpha}(t)$ and of the transcription patterns $G_{i\alpha}$ are determined from the measured values of the expression levels $X_i(t)$ of different genes.

The steady-state pattern and the patterns of the disease are distinct from one another (Box 2). Technically we express this dissimilarity by a sum, over all transcripts, of the respective weights. The same considerations also imply that the $G_{i\alpha}$ weights of the individual transcripts in pattern α are inherently standardized (Box 2).

Surprisal analysis uses the same core assumption that can be applied to characterize time-evolving physico-chemical systems. We illustrate this hypothesis here by reference to a system of coupled chemical reactions (e.g., metabolism). When such a system is initiated, reactions take place, and the concentrations of different species change. At any instant we can freeze the evolving system by adding or removing a catalyst (e.g., an enzyme) so that the system remains at its current composition. Upon such freezing, reactions stop, and the system is stable. Therefore for each transcript we can determine the work required to bring the expression to its value in the frozen state. This calculation gives us a quantitative value for each transcript.

Free Energy and the Expression Level of a Transcript. Surprisal analysis determines the free energy of the transcripts in the cell and the changes caused by the work done by the biological process. Free energy is measured in units of work or, equivalently, of energy. The fold value is a dimensionless number. The steady-state fold value of a transcript, $\ln X_i^0 = -G_{i0}\lambda_0$ (Box 1), is the thermodynamic free energy measured in units of kT per transcript where k is Boltzmann's constant, and T is the temperature. The free energy is the (reversible) work required to bring the level of expression to its steady-state value. A negative value means that the transcript is very stable, because a lot of work is needed to change the level.

The biological process does work, and, using the point of view of a frozen equilibrium we can identify the work done on the genome at each time point t . The fold value of a transcript is the work required to bring the level of expression to its present value. Thus the change in fold value between two time

points is the work done by the biological process. Quantitatively (*Experimental Procedures*, Box 1), $G_{ia}^{\lambda\alpha}(t)$ is the work required by disease transcription pattern α to bring transcript i from the steady state to its expression level at the time t .

Box 2. Transcription Patterns Are Distinct. The different transcription patterns of the same process are distinct from the steady state. Technically, when summed over all transcripts, the weights are orthogonal: $\sum_i G_{i0} G_{ia} = 0$. Also, different transcription patterns of the same process are distinct from one another: $\sum_i G_{ia} G_{i\beta} = 0, \alpha \neq \beta$. The weights are normalized: $\sum_i G_{ia} G_{ia} = 1, \alpha = 0, 1, 2, \dots$, ensuring that the values of the weights are bounded. The value of all of these sums follow because the weights of the transcripts are components of eigenvectors of a symmetric matrix that is determined from the microarray data (22, 23).

Box 3. Different Patients with the Same Symptoms Can Have Quite Different Patterns of the Disease. $G_{ia}^{(n)}$ where i is the variable is a linear array comprised of many components (the number of transcripts). The value of each component is the expression level at the pattern α for all transcripts for a particular patient n . The overlap of two different arrays is $\sum_i G_{ia}^{(n)} G_{i\beta}^{(m)}$ where the summation is over all transcripts. In vector algebra the overlap is known as the “scalar product.” It is the cosine of the angle between the two directions.

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