Supplementary Note 1. Computational Details.

A stochastic model was implemented to simulate Cap and IRES activation, ribosome initiation, elongation, termination, and potential ribosome recycling mechanisms for cap-dependent and IRES-mediated genes.

In the mathematical model, initiation events are dictated by the mRNA state. Specifically, four possible mRNA activation states were proposed (S_{OFF}, S_{CAP}, S_{IRES}, $S_{CAP-IRES}$), where: S_{OFF} represents a non-permissive initiation state; S_{CAP} allows for only cap-dependent ribosomal initiation; S_{IRES} allows for only IRES-mediated initiation; and $S_{CAP-IRES}$ allows both cap-dependent and IRES-mediated initiation. Eq. 1 represents the transition reactions between mRNA states.

$$\begin{array}{ccc} S_{OFF} & \underset{k_{OFF-C}}{\overset{k_{ON-C}}{\rightleftarrows}} & S_{CAP} \\ k_{ON-I} \downarrow\uparrow k_{OFF-I} & & k'_{ON-I} \downarrow\uparrow k'_{OFF-I}, \\ S_{IRES} & \underset{k'_{OFF-I}}{\overset{k'_{ON-I}}{\rightleftarrows}} & S_{CAP-IRES} \end{array} \qquad (1)$$

where each k_x represents a first-order transition rate between two RNA states, and k'_x is the transition rate conditioned on the activation state of the other construct (e.g., k'_{ON-I} is the cap-dependent activation rate of IRES). A simpler three-state model was considered by removing the fourth RNA state (i.e., $S_{CAP-IRES}$). The parameter estimation section describes how a system with three or four mRNA states was chosen.

When the system is in one of the appropriate mRNA activity states, cap-dependent and IRES-mediated initiation events occur with propensities w_{INIT-C} and w_{INIT-I}, respectively, which are defined:

$$w_{INIT-C} = \begin{cases} k_{INIT-C}, & if\ S_{CAP}\ or\ S_{CAP-IRES}\ , \\ 0, & otherwise, \end{cases} \qquad (2)$$

$$w_{INIT-I} = \begin{cases} k_{INIT-I}, & if\ S_{IRES}\ or\ S_{CAP-IRES}\ , \\ 0, & otherwise, \end{cases} \quad (3)$$

where k_{INIT-C} and k_{INIT-I} represent the cap and IRES initiation rates, respectively.

To simulate the model under stochastic dynamics, Eqs. (2) and (3) were used to generate a vector of random initiation event times for each gene, $\tau_{INIT_{IRES}}$ and $\tau_{INIT_{CAP}}$. A codon-dependent model for translation was used, in which the elongation rate for each codon is given by $\bar{k}_e(^{u_i}/_{\bar{u}})$, where u_i is the known frequency of the i^{th} codon in the human genome, $\bar{u}$ is the average codon usage frequency in the human genome, and $\bar{k}_e$ is the basal elongation rate (to be estimated from the data). In the models, the final codon termination rates were assumed to be equal to the average elongation rate.

For increased computational efficiency, ribosome elongation was approximated using a coarse-grained procedure. For this, sparse ribosome loading was assumed to enable simple calculation of the average time needed by a ribosome to complete gene elongation, τ_{k_e}, as follows:

$$\tau_{ke} = \sum_{i=1}^{L} \frac{1}{\bar{k}_e\left(^{u_i}/_{\bar{u}}\right)}, \quad (4)$$

where L represents the gene length in codons. Using the specific gene sequence for the cap-dependent gene and IRES-mediated gene, we calculated the total elongation time τ_{Cap} and τ_{IRES}, respectively. At any time, t, such that $0 < t - \tau_{INIT_{Cap}} < \tau_{Cap}$, the position of a given cap-translating ribosome was obtained by calculating the proportion of elongated gene as follows:

$$x_{CAP} = j \text{ such that } \sum_{i=1}^{j} \frac{1}{\bar{k}_e\left(^{u_i}/_{\bar{u}}\right)} \leq t - \tau_{INIT_{CAP}} < \sum_{i=1}^{j+1} \frac{1}{\bar{k}_e\left(^{u_i}/_{\bar{u}}\right)}\ , \quad (5)$$

and for the IRES-mediated gene for $0 < t - \tau_{INIT_{IRES}} < \tau_{IRES}$:

$$x_{IRES} = j \text{ such that } \sum_{i=1}^{j} \frac{1}{\bar{k}_e\left(u_i/\bar{u}\right)} \leq t - \tau_{INIT_{IRES}} < \sum_{i=1}^{j+1} \frac{1}{\bar{k}_e\left(u_i/\bar{u}\right)}\ , \qquad (6)$$

where $\tau_{INIT_{CAP}}$ and $\tau_{INIT_{IRES}}$ are the times at which the corresponding ribosome initiated translation begins.

To consider potential interaction mechanisms between cap-dependent and IRES-mediated translation, two possible hypotheses were postulated:

A first hypothetical model considers potential ribosome recycling (or crossover) mechanisms, by which a ribosome that completes translation of the cap-dependent gene could immediately re-initiate translation of the IRES-mediated gene. In this context, a new propensity, w_{CI}, that specifies the probability that a ribosome completing cap will re-initiate at IRES was introduced. The specification of such reactions reflects single-mRNA translation observations by Wang et al., 2016[1], which suggest ribosome hops between adjacent open reading frames on a single RNA. To test if such recycling mechanisms are necessary to reproduce the experimental data, multiple models with and without nonzero values for the crossover rate w_{IC} were compared.

In the second hypothetical model, cap and IRES interdependency were tested by assuming that the activation and deactivation of cap or IRES could depend on the activity state for the other sensor (e.g., IRES could activate faster when cap is already active). Including different combinations of these hypothetical mechanisms in the three- and four-state models led us to propose a list of 14 different sub-models, each comprising between 7 and 12 free parameters (see Extended Data Fig. 6b). The sub-models test different hypotheses, including variations of the number of mRNA states (3 or 4 states), dependency on Cap and IRES switching states, and/or the existence of the cross-over mechanism. Cap and IRES dependency

are represented in the see Extended Data Fig. 6b by red lines, which denote that the corresponding reaction parameter value has a free value during the optimization process. All models have 3 or 4 mRNA states, denoted by 3S or 4S, respectively. From see Extended Data Fig. 6b left to right, the first seven models lack crossover, while the last seven have cross-over (denoted by subscript 'C', e.g. 3SC). Models can have independent (denoted by subscript 'I') or dependent (denoted by subscript 'D') Cap or IRES activation/deactivation. Models can also have a single dependent activation or deactivation rate (denoted by subscript 'm1' or 'm2').

Converting ribosome elongation times to fluorescence intensity

To relate the ribosome elongation times to fluorescence intensity, a similar approach as in Aguilera et al.[2] was adopted. Ribosome occupancy is converted to fluorescence intensity by increasing the simulated intensity by one unit after each ribosome moves across the tag-region. For this, a cumulative *probe design vector* was defined that records the number of probe sites upstream from each codon, $\boldsymbol{c_g} = [c_1, c_2, \ldots, c_L]$, for the appropriate construct (i.e., g = cap-dependent or IRES-mediated genes, respectively). Using this, the intensity was calculated as the sum of the product of the position of the ribosome at a given time and $\boldsymbol{c_g}$. For cap-dependent spots, the intensity vector is defined as:

$$I_{CAP}(t) = \sum \boldsymbol{c}_{CAP}(\boldsymbol{x}_{CAP}(t)), \quad (8)$$

and for IRES-mediated spots it is:

$$I_{IRES}(t) = \sum \boldsymbol{c}_{IRES}(\boldsymbol{x}_{IRES}(t)), \quad (9)$$

where $\boldsymbol{c}_g(\boldsymbol{x}_g)$ is the intensity of a given ribosome at position $\boldsymbol{x}_g$, and the summations are taken over all ribosomes present on the mRNA at time t. To have consistent units of intensity between model simulations and experimental data, intensity values are reported in units of mature proteins (u.m.p.) as described in detail on the Methods section.

Comparison of experimental data and model

To reproduce experimental data, the model was simulated using a modified Direct Method[3] for 4000 trajectories representing independent RNA spots. Simulations were run for a burn-in period of 10,000 seconds to approximate steady state. Simulations were processed and used to capture spot intensity for the cap-dependent gene (I_{CAP}) and the IRES-mediated gene (I_{IRES}). Additionally, simulated spots were classified as cap-dependent with probability P_{CAP}, IRES-mediated with probability P_{IRES}; both with probability $P_{CAP-IRES}$, or neither with probability P_{None}.

Modeling Harringtonine experiments

Harringtonine inhibits new initiation events by directly blocking the 60S subunit in the ribosome, and it has been widely used to perform run-off assays to estimate elongation rates.[4] To mimic the effects of Harringtonine in our model, the initiation rate was modified for the first gene as follows:

$$w_{INIT-C} = \begin{cases} k_{INIT-C}, & if\ t < t_H\ , \\ 0, & otherwise, \end{cases} \tag{10}$$

and the initiation rate for the second gene as follows:

$$w_{INIT-I} = \begin{cases} k_{INIT-I}, & if\ t < t_H\ , \\ 0, & otherwise, \end{cases} \tag{11}$$

where t_H is the time of application of Harringtonine.

Modeling Sodium Arsenite (NaAs) and Dithiothreitol (DTT) experiments

NaAs and DTT are chemical stresses that have been used to affect cap-dependent initiation in previous single-molecule translation experiments.[1] The mechanism of action for NaAs is not well understood, but it has been suggested to affect ribosome initiation through its

action on translation factors, such as eIF2a and eIF4.[5] To simulate these chemical stresses, two potential mechanisms of action were tested. The first potential mechanism of action involves blocking cap-dependent translation by affecting its RNA state, and was implemented in the model by modifying the cap activation rates, k_{ON-C} and k'_{ON-C}, as follows:

$$k_{ON-C} = \begin{cases} k_{ON-C}, & if\ t < t_{ST}, \\ k_{ST} \cdot k_{ON-C}, & otherwise, \end{cases} \quad (12)$$

and

$$k'_{ON-C} = \begin{cases} k'_{ON-C}, & if\ t < t_{ST}, \\ k_{ST} \cdot k'_{ON-C}, & otherwise, \end{cases} \quad (13)$$

where, k_{ST} is an inhibition constant, where a total inhibition is achieved by $k_{ST} = 0$, and a null inhibition is achieved by $k_{ST} = 1$. The time t_{ST} denotes the time of stress application.

In the second mechanism of action, it was hypothesized that the drug directly blocks cap-dependent translation initiation. In the model, this is achieved by modifying w_{ini} as follows:

$$w_{INIT-C} = \begin{cases} k_{INIT-C}, & if\ t < t_{ST}, \\ k_{ST} \cdot k_{INIT-C}, & otherwise. \end{cases} \quad (14)$$

Parameter estimation and optimization routines

The parameter estimation strategy consists of finding a parameter set ($\overline{\Lambda}$) that statistically reproduces all experimental data, including intensity histograms, fractions of translating spots, and Harringtonine ribosomal run-off assays as follows:

Intensity histograms

To compare experimental and simulated steady-state intensity histograms, the probability to observe the experimentally determined intensities (d^{cap} or d^{IRES}) was estimated

given a parameter set (Λ) in the model implementation. To estimate $P(d;\Lambda)$, histograms were collected using $N_t = 4000$ independent stochastic trajectories per parameter evaluation. The likelihood function was estimated as follows:

$$L_{Dist}(D|M) = \prod_{j=1}^{N_D} P(d_j;\Lambda), \quad (15)$$

and the log-likelihood as:

$$\log L_{Dist}(D|M) = \sum_{j=1}^{N_D} \log P(d_j;\Lambda), \quad (16)$$

where D represents the data measured in N_D independent experimental data, and M corresponds to the model. As the experimental measurements can only detect protein intensities above a threshold of one mature protein, all spots with intensities below 1 u.m.p. were defined as non-translating mRNA. This metric was applied to experimental data consisting of cap-dependent spots (CAP) and IRES-mediated spots (IRES). With this, a total log-likelihood function was calculated as the sum of the functions for cap and IRES spots, that is:

$$\log L_{T_{Dist}}(D|M) = \sum_{j=1}^{N_t} \log P_{CAP}\left(d_j^{cap};\Lambda\right) + \sum_{j=1}^{N_t} \log P_{IRES}\left(d_j^{IRES};\Lambda\right). \quad (17)$$

Fraction of translating spot

A similar approach was used to compute the likelihood to observe the experimentally determined number of spots classified as Cap-only, IRES-only, Cap+IRES, and non-translating. The likelihood function was computed as follows:

$$L_F(D|M) = \prod_{j=1}^{N_D} P(f_j;\Lambda), \quad (18)$$

and the log-likelihood as:

$$\log L_F(D|M) = \sum_{j=1}^{N_D} \log P(f_j;\Lambda) = \sum_i N_i \log P(f_i;\Lambda), \quad (19)$$

where each f_j denotes the type (i.e., Cap, IRES, Cap+IRES, or non-translating) of the j^{th} spot, N_D is the total number of independent observed spots, N_i is the number of independent observed spots of the i^{th} type, and $P(f_j;\Lambda)$ is the categorical distribution of spots of each type estimated by the model simulations with parameters Λ.

Harringtonine induced ribosomal run-off

To compare simulated and experimental time course data representing the intensity after Harringtonine application, a Gaussian likelihood function was assumed and calculated as follows:

$$L_{HT}(I_D|I_M) = \prod_{i=1}^{N_D} \frac{1}{\sqrt{2\pi\sigma(t_i)^2}} \exp\left(-\frac{\left(I_D(t_i)-I_M(t_i;\Lambda)\right)^2}{2\sigma(t_i)^2}\right), \quad (20)$$

with a log-likelihood form given by:

$$\log L_{HT}(I_D|I_M) = C_{HT} - \sum_{i=1}^{N_D} \frac{\left(I_D(t_i)-I_M(t_i;\Lambda)\right)^2}{2\sigma(t_i)^2}, \quad (21)$$

where $\sigma(t_i)$ is approximated by the measured SEM, and N_D is the number of time points from the Harrintonine run-off curve. In this log-likelihood formulation, C_{HT} is a constant that doesn't depend on the parameters.

Experimental data was quantified for the total intensities for cap (I_{CAP-D}) and IRES (I_{IRES-D}) within all spots (after subtraction of the base level of intensity). These two data sets were collected to compute a total log-likelihood function as follows:

$$\log L_{T_{HT}}(I_D|I_M) = \log L_{HT_{CAP}}(I_{CAP-D}|I_M) + \log L_{HT_{IRES}}(I_{IRES-D}|I_M). \quad (22)$$

Parameter searches consisted of optimization routines based on genetic algorithms (GA) using the function *ga* in MATLAB. The optimization routine was implemented with a population of 100 individuals for 30 generations, and the implementation was run multiple times with random initial

conditions. Additionally, the Pattern Search Algorithm[6] was implemented using the function *patternsearch* in MATLAB to ensure convergence. The best parameter values were selected by minimizing a global objective function that considers all data sets, that is:

$$-\log L_{Total}(D|M) = -\big(\log L_{T_{Dist}}(D|M) + \log L_F(D|M) + \log L_{T_{HT}}(I_D|I_M)\big). \quad (23)$$

The comparison of the optimization results for all tested models is given in Extended Data Fig. 6c-d.

***Assessing how well models predict Sodium Arsenite (NaAs) and Dithiothreitol (DTT)* experiments**

After optimizing the models, cross-validation experiments were predicted using the chemical stresses, NaAs and DTT. For this, simulated and experimental time course data representing the total translation spot intensity after NaAs or DTT application were compared. The likelihood function was calculated as follows:

$$L_{ST}(I_D|I_M) = \prod_{i=1}^{N_D} \frac{1}{\sqrt{2\pi\sigma(t_i)^2}} \exp\left(-\frac{\left(I_D(t_i) - I_M(t_i;\Lambda)\right)^2}{2\sigma(t_i)^2}\right), \quad (24)$$

and the log-likelihood function is:

$$\log L_{ST}(I_D|I_M) = C_{ST} - \sum_{i=1}^{N_D} \frac{\left(I_D(t_i) - I_M(t_i;\Lambda)\right)^2}{2\sigma(t_i)^2}, \quad (25)$$

where $\sigma(t_i)$ is approximated by the measured SEM, and N_D is the number of time points measured in the drug-treatment curve, and C_{ST} is constant that doesn't depend on model parameters.

For chemical stress experiments, three data sets were used representing the intensity for Cap-only spots, IRES-only spots, and green (Cap) intensity in both cap and IRES spots. These three data sets were considered on a total log-likelihood function as follows:

$$-\log L_{T_{ST}}(I_D|I_M) = -\left(\log L_{ST_{CAP}}(I_{CAP-D}|I_M) + \log L_{ST_{IRES}}(I_{IRES-D}|I_M) + \log L_{ST_{CI}}(I_{Cap+IRes-D}|I_M)\right). \quad (26)$$

Uncertainty Quantification

To quantify uncertainty, the best parameter set from fitting was initially used and 100 runs of 1,000 step Markov Chain Monte Carlo (MCMC) algorithm were run to explore an additional 100,000 possible parameter combinations. At each step, a random perturbation of 10% to the current parameters was proposed, and every proposal for which the log-likelihood for the new parameter set was within a 1% of that found for the best fit was accepted (i.e., all parameters for which $\log(L(I_D|I_{Best})/L(I_D|I_{New})) < 60$ were accepted). The standard deviation of the resulting 26,650 accepted parameter sets was then used as a measure of parameter uncertainty as shown in Table 1.

Computational Implementation and Codes

All simulations were performed on the W. M. Keck High Performance Computing Cluster at Colorado State University. All codes and required data are available at: https://github.com/MunskyGroup/Koch_Aguilera_etal_2020.git.

Supplementary Note References

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