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SUPPLEMENTARY MATERIALS

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AGING CELL BIOLOGY

Life-span extension by a metacaspase in the yeast *Saccharomyces cerevisiae*

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Single-cell species harbor ancestral structural homologs of caspase proteases, although the evolutionary benefit of such apoptosis-related proteins in unicellular organisms is unclear. Here, we found that the yeast metacaspase Mca1 is recruited to the insoluble protein deposit (IPOD) and juxtanuclear quality-control compartment (JUNQ) during aging and proteostatic stress. Elevating *MCA1* expression counteracted accumulation of unfolded proteins and aggregates and extended life span in a heat shock protein Hsp104 disaggregase– and proteasome-dependent manner. Consistent with a role in protein quality control, genetic interaction analysis revealed that *MCA1* buffers against deficiencies in the Hsp40 chaperone *YDJ1* in a caspase cysteine–dependent manner. Life-span extension and aggregate management by Mca1 was only partly dependent on its conserved catalytic cysteine, which suggests that Mca1 harbors both caspase-dependent and independent functions related to life-span control.

The cysteine-dependent aspartate-directed proteases, or caspases, are a family of proteases required for apoptosis [programmed cell death (PCD)] (*1*–*3*). PCD is vital for proper development, tumor suppression, immunity, and neuron homeostasis (*4*). The yeast *Saccharomyces cerevisiae* expresses a single, type I metacaspase [ancestral structural caspase homolog (*5*)] called Mca1 (Yca1) (*6*). After particular stresses, *S. cerevisiae* display numerous markers of PCD, dependent on the presence of Mca1 (*6*–*8*). Although these results imply that Mca1 is a PCD executioner protein, other reports suggest that Mca1 has beneficial functions independent of PCD, for example, in protein quality control (PQC) (*9*–*13*). PQC ensures that individual proteins are accurately produced, folded, compartmentalized, degraded, and prevented from aggregating (*14*). When, for example, during severe stress and aging, PQC fails to fully prevent protein aggregation, a second line of defense, spatial PQC, ensures that aggregates are not equally inherited during cytokinesis, a phenomenon that resets age in one cell lineage (*15*–*19*). In the budding yeast *S. cerevisiae*, this mother cell–biased segregation of aggregated proteins requires the heat shock protein Hsp104 (*20*, *21*).

By crossing *HSP104* fused to green fluorescent protein, *HSP104-GFP* (*22*, *23*), into a synthetic genetic miniarray of yeast mutants lacking chaperones or cochaperones and Hsp104 interactors (*16*, *22*, *24*), we showed that Mca1 is required for keeping yeast daughter cells free of aggregated proteins generated during a transient heat shock (fig. S1A). Constitutively elevating Mca1 levels (fig. S1B) by exchanging the weak *MCA1* promoter with the strong promoter (P_{GPD}) of the *GPD* gene boosted aggregate asymmetry during cytokinesis (fig. S1A). Asymmetric aggregate distribution can be achieved by limiting inheritance (retention in mother cells) or by aggregate removal (disaggregation and/or retrograde transport) in daughter cells (*16*, *24*). These two processes were experimentally distinguished using fluorescent concanavalin A (Con A) staining of the cell wall (Fig. 1, A and B), which revealed that most genes required for establishing asymmetry (fig. S1A) were important for both aggregate removal and asymmetrical inheritance (Fig. 1C). However, deletions of *MCA1* and *SSA3* only affected aggregate removal (Fig. 1C). Consistently, Mca1 overproduction enhanced aggregate removal while leaving the process of inheritance unaffected (Fig. 1D).

Using genome-wide synthetic genetic array (SGA) analysis (*23*, *25*), we next identified genes that interacted negatively with *MCA1*, which demonstrated that Mca1 buffers against deficiencies in a few discrete functions (Fig. 2A and fig. S2A), including PQC, through negative interactions with *CDC48* and *RPT1* of the proteasome system. Among the nonessential genes, only one significant interaction was found: a strong negative interaction with *YDJ1*, which encodes the major cytosolic Hsp40 chaperone (Fig. 2, A to C). In addition, removing *MCA1* in the *ydj1*Δ mutant exacerbated cell shape abnormalities (large, elongated buds) (Fig. 2D) and aggregate morphology (multiple amorphous aggregate structures rather than inclusion bodies) (Fig. 2E and fig. S2, B and C). Like metazoan caspases, Mca1 contains a conserved active-site cysteine [cysteine C276, previously designated C297 (*6*, *26*)], and exchanging this single cysteine to alanine was sufficient to cause a negative genetic interaction with *ydj1*Δ (Fig. 2F).

In line with the suggested association of Mca1 with aggregates (*11*), Mca1 relocalized to Hsp104-associated cytosolic puncta during both heat stress (fig. S3A) and aging (Fig. 3A). We used the temperature-sensitive variant of the SUMO-conjugating enzyme, $Ubc9^{ts}$-RFP, with red fluorescent protein (RFP), as a marker for juxtanuclear quality-control compartment (JUNQ) and the Hsp104-enriched insoluble protein deposit (IPOD) inclusions (*17*, *27*). We found that Mca1 associated with aggregates within both these compartments (Fig. 3B) and that the JUNQ association (fig. S3B) was often close to the nucleolus (Fig. 3C) (*28*). During stress and aging, Mca1 relocated to inclusions independently of Ydj1, Hsp104, and Ssa1 and 2 (Fig. 3D and fig. S3C). Further, cells without Mca1, like cells lacking Ydj1, displayed increased numbers of inclusion in aged cells (Fig. 3, E and F), and age-related accumulation of inclusions was counteracted in both wild-type and *ydj1*Δ cells by overproducing Mca1, albeit to a higher degree in wild-type cells (Fig. 3F). The IPOD-specific reporter Rnq1 (*27*) revealed that most aggregates in old cells were IPODs and that Mca1 overproduction caused a 30% reduction in the accumulation of these inclusions (fig. S3, D and E). To test by which route Mca1 may prevent the buildup of such protein inclusions, we determined the effect of Mca1 dosage on luciferase folding (*29*) and the levels of the signal sequence–deficient, unfolded carboxypeptidase Y,

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Fig. 1. Mca1 dosage affects the establishment of aggregate asymmetry during cytokinesis. The aggregate reporter Hsp104-GFP was used to test if the genes identified as being required for establishing aggregate asymmetry (fig. S1A) during cytokinesis were doing so by preventing aggregate inheritance or by boosting aggregate removal in daughter cells. This analysis established that Mca1 is limiting for aggregate removal in yeast daughter cells. (**A**) Schematic overview of the Con A staining protocol to discriminate between aggregate inheritance and/or retention (unstained buds, produced after heat shock) and removal (stained buds, present during heat shock). (**B**) Experimental examples depicting aggregate removal (left) and retention (right). Top rows show successful events (+), bottom rows exemplify failed processes (−), generating daughter cells containing aggregate(s). Scale bar, 5 μm. (**C**) Defects in aggregate removal and/or retention in asymmetry mutants identified in fig. S1A. Data are plotted as the mutants' deviation from the wild type (WT) in aggregate inheritance (white bars; 100% increase means a twofold increase in mutant daughter cell inheritance compared with WT daughters) and removal (black bars; –100% means a twofold decrease in mutant daughter cell removal of aggregates compared with WT daughters). Data are means ± SD. $N = 3$ for *ssa1*Δ, *ssa2*Δ, *ssa3*Δ, *ssb1*Δ, *hsp82*Δ; $N = 4$ for *ydj1*Δ; $N = 5$ for *mca1*Δ. (**D**) Aggregate removal and inheritance in the Mca1-overproducing strain (P_{GPD}-*MCA1*). Data are means ± SD. $N = 3$. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

CPY*, fused to Leu2 (hereafter called ΔssCL*) (*30*, *31*). We found that neither Mca1 overproduction nor an *mca1*Δ deletion affected folding activity (fig. S3F). In contrast, Mca1 overproduction reduced, whereas *mca1*Δ increased, the levels of the unfolded proteasome substrate ΔssCL* (Fig. 3, G and H), which suggested that Mca1 is required for efficient removal of terminally unfolded proteins.

In view of these data, we tested whether *MCA1* was acting as a pro- (executioner) or anti- (gerontogene) aging gene. The lack of Mca1 had little effect on the replicative life span in otherwise wild-type cells but accelerated aging of cells lacking Ydj1 (Fig. 4A), which confirmed that Mca1 becomes indispensable in the absence of Ydj1. Overproducing Mca1 extended life span (46 to 56%) in a manner partly dependent on Ydj1 (Fig. 4, B and C). That life-span extension by Mca1 overproduction was less efficient in cells lacking Ydj1 is consistent with a more pronounced reduction of aggregates in the former (Fig. 3F) and suggests that proper protein homeostasis is required to achieve full effects of elevated Mca1 levels. Indeed, overproduction of Mca1 did not result in a statistically significant extension of life span in the absence of Hsp104 (Fig. 4D) or a reduction in proteasome levels by deleting the proteasome regulator Rpn4 (*32*) (Fig. 4E). Thus, the removal of unfolded and aggregated proteins is a key feature of Mca1's role in life-span control.

The conserved catalytic cysteine-histidine dyad of Mca1 is, like metazoan caspases, activated under certain conditions including H_2O_2 exposure, to autocatalytically remove a small ~12-kD subunit on the Mca1 protein (*6*, *33*). We found that this 12-kD subunit accumulated in aged cells carrying the P_{GPD}-*MCA1* fusion, similar to cells exposed to H_2O_2 (Fig. 4F), which indicated that typical metacaspase processing is triggered upon mother cell aging. The $Mca1_{C276A}$ protein was, when overproduced, incapable of removing the 12-kD subunit during aging (Fig. 4F) and partially defective in counteracting aggregate accumulation (Fig. 4G) and extending life span (Fig. 4H). Overproduction of $Mca1_{C276A}$ in cells lacking Ydj1 reduced rather than extended life span (Fig. 4I), consistent with the requirement for the active-site cysteine in Ydj1-deficient cells (Fig. 2F). Thus, both cysteine-dependent and independent functions of Mca1 are required for life-span control of which the cysteine-independent one can only be realized in cells harboring functional Ydj1.

Here, we have shown that Mca1 has a beneficial cell-autonomous function in PQC that can compensate for the lack of the major Hsp40 chaperone of the yeast cytosol and facilitate the removal of an unfolded protein, counteract accumulation of protein aggregates, and prolong cellular life span in an Hsp104 disaggregase- and proteasome-dependent manner. The fact that the Hsp104 disaggregase is required for Mca1 to extend life span

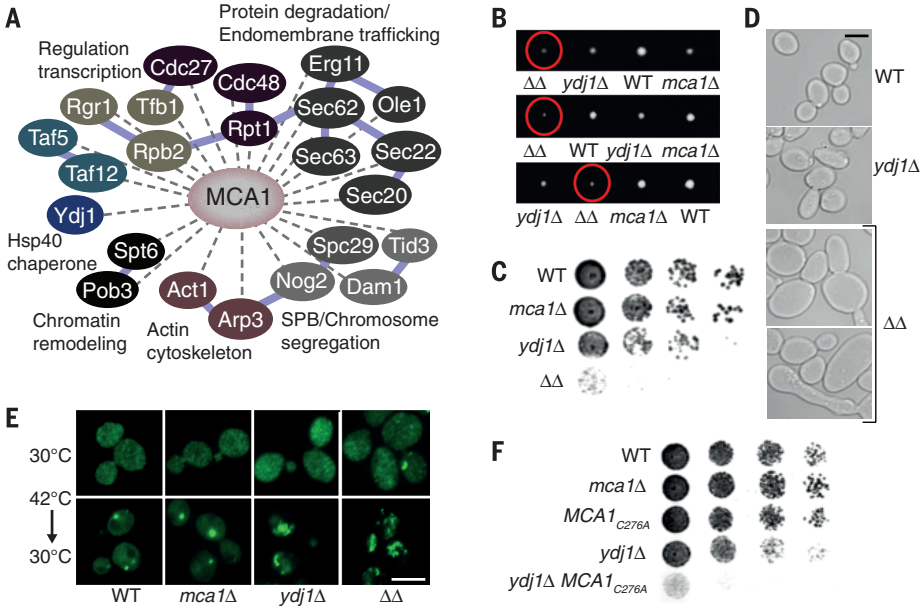


Fig. 2. *MCA1* displays negative genetic interactions with *YDJ1* and genes involved in PQC. To identify a possible function of Mca1 in PQC, the genetic interaction network of *MCA1* was determined for both essential and nonessential genes, which demonstrated that Mca1 is buffering against deficiencies in the major Hsp40 chaperone, Ydj1, and components of the proteasome. (**A**) Negative genetic interactions of *MCA1*, clustered in functional groups, connected by known physical interactions (purple lines). (**B**) Growth of spores after reconstructing *ydj1*Δ *mca1*Δ (ΔΔ, red circle) double mutants by genetic crossing. (**C**) Growth defect of a reconstructed *ydj1*Δ *mca1*Δ (ΔΔ) double mutant. (**D**) Cell morphologies of the WT, *ydj1*Δ, and *ydj1*Δ *mca1*Δ (ΔΔ) strains. (**E**) Aggregate morphology after heat treatment in WT, *ydj1*Δ, *mca1*Δ, and *ydj1*Δ *mca1*Δ (ΔΔ), visualized by Hsp104-GFP. Scale bar, 5 μm. (**F**) Growth defect of a *ydj1*Δ $MCA1_{C276A}$ double mutant. $N > 2$ for all tests in (B) to (F).

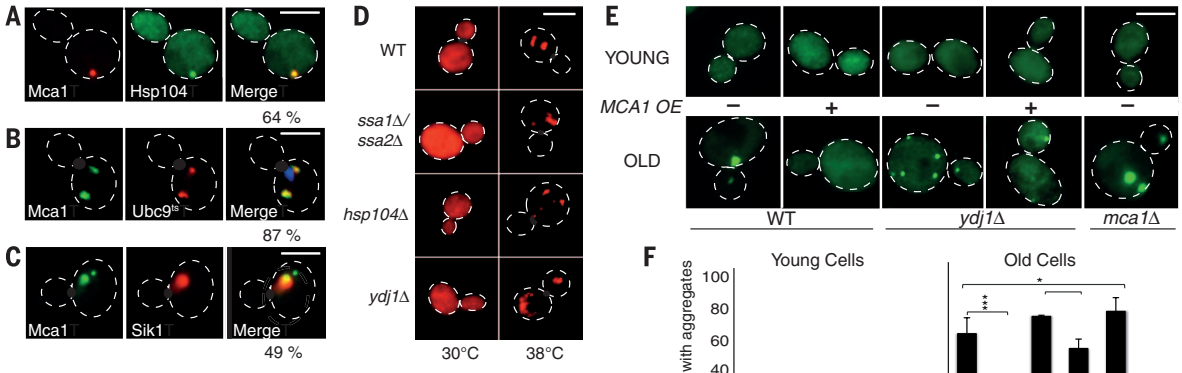


Fig. 3. Mca1 localizes to IPOD and JUNQ quality-control compartments and prevents accumulation of unfolded proteins and aggregates formed upon aging. (**A**) Colocalization of Mca1 and Hsp104 at aggregates in replicatively aged cells (12 generations). The fraction (%) of cells displaying colocalization is indicated near the micrograph; 400 cells were analyzed. $N > 2$. (**B**) Colocalization of Mca1 and Ubc9^{ts} at peripheral (IPOD) and juxtanuclear (JUNQ) compartments [4′,6′-diamidino-2-phenylindole (DAPI)–stained nucleus in blue]. The fraction (%) of Mca1 foci colocalizing with Ubc9^{ts} is indicated near the micrograph; 188 cells were analyzed. $N > 2$. (**C**) Mca1 localization to aggregates at peripheral sites and the nucleolus, visualized by Sik1-RFP. The fraction (%) of Mca1 foci colocalizing with Sik1 is indicated near the micrograph; 245 cells were analyzed. $N > 2$. (**D**) Mca1 association with aggregates in the absence of *SSA1*, *SSA2*, *HSP104*, and *YDJ1* as indicated. $N > 2$. (**E**) Visualization of Hsp104-GFP aggregates in young and replicatively aged cells as indicated with (+) and without (−) overexpression of *MCA1* (*MCA1 OE*). Scale bars, 5 μm. (**F**) Percentage of cells from (E) containing aggregates in young (0 generations) and aged cells (13 generations for WT and *mca1*Δ, 9 generations in *ydj1*Δ strains). $N = 6$ for *mca1*Δ and WT *MCA1 OE*, $N = 3$ for *ydj1*Δ and *ydj1*Δ *MCA1 OE*. Data are means ± SD. $^{*}P < 0.05$; $^{***}P < 0.0005$. (**G**) The ΔssCL* protein that misfolds in the cytoplasm and is degraded by the proteasome (*30*, *31*) was used to test if Mca1 is involved in facilitating removal of misfolded proteins. Cells expressing ΔssCL* (ΔssCPY* fused to Leu2) were spotted on indicated media and incubated for 3 days at 30°C. Enhanced growth on plates lacking leucine indicates stabilization of ΔssCL* (*30*, *31*), whereas reduced growth indicates increased degradation. $N = 3$. (**H**) Immunoblotting showing relative ΔssCL* levels of the indicated strains. Data are means ± SD. $N = 3$.

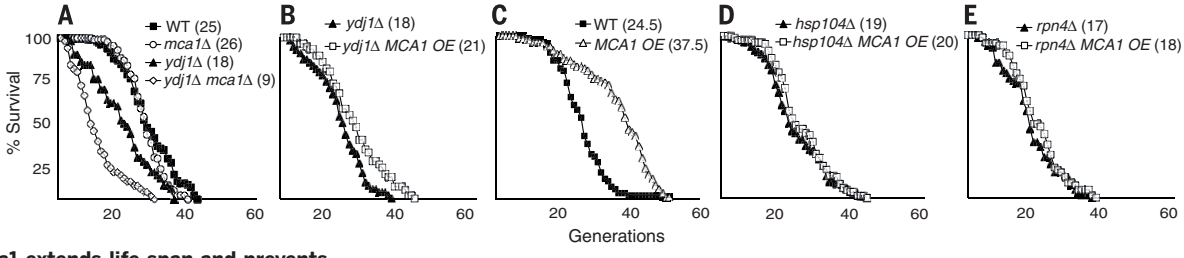
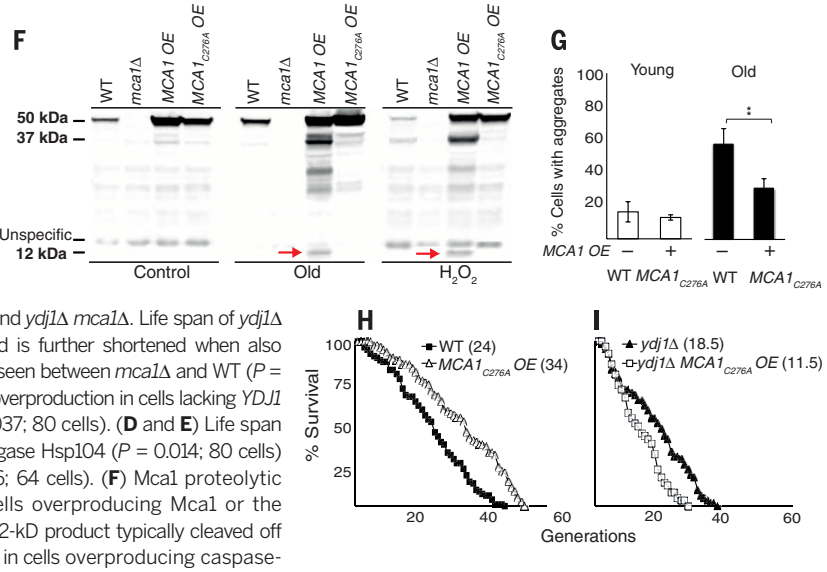


Fig. 4. Mca1 extends life span and prevents aggregate accumulation in a partially caspase cysteine–independent manner. To test if Mca1 acts as an executioner gene or gerontogene during the life history of yeast mother cells, the replicative life span of Mca1-deficient and Mca1-overproducing cells was determined. This analysis demonstrated that Mca1 acted as a life-span–extending gene and that life-span extension required both Hsp104-dependent disaggregase activity and fully functional proteasomes linking Mca1-dependent life-span control to the removal of damaged and aggregated proteins. (**A**) Replicative life span of WT, *ydj1*Δ, *mca1*Δ, and *ydj1*Δ *mca1*Δ. Life span of *ydj1*Δ is shorter than that of WT (P = 5.0E-4; 50 cells) and is further shortened when also deleting *MCA1* (P = 5.1E-5; 50 cells). No difference was seen between *mca1*Δ and WT (P = 0.99; 80 cells). (**B** and **C**) Life-span extension by Mca1 overproduction in cells lacking *YDJ1* (B) (P = 3.00E-2; 80 cells), and in WT cells (C) (P = 0.037; 80 cells). (**D** and **E**) Life span of the Mca1-overproducing strain lacking the disaggregase Hsp104 (P = 0.014; 80 cells) (D) and the proteasome regulator Rpn4 (E) (P = 0.16; 64 cells). (**F**) Mca1 proteolytic processing during aging and H_2O_2 exposure in cells overproducing Mca1 or the caspase-inactive Mca1$_{C276A}$. Red arrow indicates the 12-kD product typically cleaved off upon caspase activation. (**G**) Age-induced aggregation in cells overproducing caspase-inactive Mca1$_{C276A}$. Data are means ± SD. $N = 3$. $^{**}P < 0.005$. (**H** and **I**) Effect of overproducing the caspase-inactive Mca1$_{C276A}$ on the life span of WT (H) (P = 3.5E-5; 64 cells) and *ydj1*Δ (I) (P = 0.0082; 64 cells) cells.



suggests that protein aggregates and/or inclusions are true aging factors in the yeast model system. Because the beneficial functions of Mca1 related to aggregate management and longevity are only partially dependent on the catalytic cysteine C276, it is conceivable that under some conditions, metacaspase-dependent PCD activities (*6*–*8*) may be balanced by both metacaspase-dependent and independent proteostasis. In addition, the data raise the possibility that caspases and/or metacaspases originally evolved as PQC-related cytoprotective factors that were later adopted as PCD-related executioners, perhaps upon their overinduction during severe stress. Elucidating the nature of the environmental cues regulating the switching of metacaspase functions between proteostasis and PCD might explain how decisions concerning survival are made at the level of the individual cell versus the cell community.

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SUPPLEMENTARY MATERIALS

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HIV LATENCY

Screening for noise in gene expression identifies drug synergies

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Stochastic fluctuations are inherent to gene expression and can drive cell-fate specification. We used such fluctuations to modulate reactivation of HIV from latency—a quiescent state that is a major barrier to an HIV cure. By screening a diverse library of bioactive small molecules, we identified more than 80 compounds that modulated HIV gene–expression fluctuations (i.e., "noise"), without changing mean expression. These noise-modulating compounds would be neglected in conventional screens, and yet, they synergized with conventional transcriptional activators. Noise enhancers reactivated latent cells significantly better than existing best-in-class reactivation drug combinations (and with reduced off-target cytotoxicity), whereas noise suppressors stabilized latency. Noise-modulating chemicals may provide novel probes for the physiological consequences of noise and an unexplored axis for drug discovery, allowing enhanced control over diverse cell-fate decisions.

From infectious disease to stem cells and cancer, therapeutic manipulation of cell fate remains a fundamental goal. Efficient manipulation has proven difficult, in part, because cell-fate decisions are often regulated by stochastic cellular processes (*1*–*4*) that generate heterogeneity in signaling responses and result in substantial cell-to-cell variability. For pathogens that establish persistent states (e.g., HIV latency), therapeutic targeting and perturbation of the dormant-cell phenotype has proven exceptionally challenging.

HIV can enter a long-lived proviral latent state (Fig. 1A) that is a leading obstacle to a cure (*5*) and requires that infected individuals remain on lifelong antiretroviral therapy. A leading HIV-cure strategy attempts to stimulate the latent virus back into an active-replication state and simultaneously eliminate it by antiretroviral therapy (*6*). However, efficient reactivation of latent HIV has had limited success (*7*), perhaps because of the stochastic nature of latency (*8*–*11*).

Small-molecule compounds that promote HIV reactivation have been identified, primarily from reporter assays that detect amplification of the mean level of HIV gene expression (*12*), but these compounds fall short of completely reactivating latency (*10*). However, given the stochastic nature of gene expression (*13*), the mean represents only one aspect of expression. We examined whether compounds that affect fluctuations around the mean gene-expression level (i.e., "noise") synergize with existing drug candidates.

Noise in gene expression often results from promoter transitions between on and off states that generate episodic "bursts" of transcription (*13*–*17*). In this "two-state" model, RNA polymerase II stalls on the HIV long terminal repeat (LTR) promoter (*18*, *19*), but when the elongation stall is relieved, multiple polymerases can read through, which results in a burst of transcripts and highly variable expression levels (Fig. 1A, inset). If HIV expression reaches sufficient levels, HIV's autoregulatory gene products (i.e., Tat and Rev) drive reactivation and ultimately active replication. Transcriptional activators, such as tumor necrosis factor (TNF), typically activate the LTR by increasing transcriptional initiation, thereby increasing the frequency of transcriptional events (*18*, *19*) (Fig. 1B). In contrast, compounds that modulate elongation increase the

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