Supporting Information for

Fluorescence Turn-On Folding Sensor to Monitor Proteome Stress in Live Cells

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Supplementary Figures

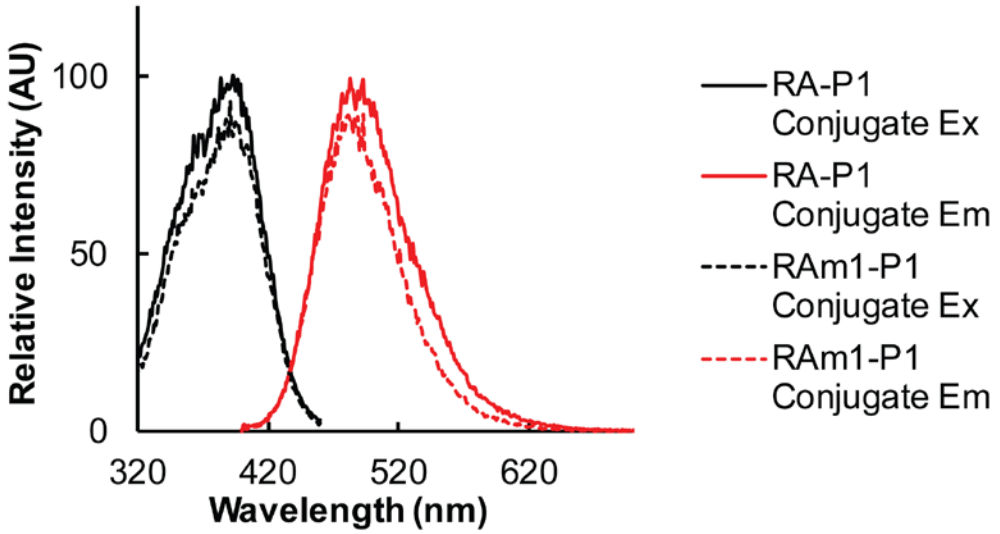

Figure S1. RA-**P1** and RAm1-**P1** conjugates exhibit very similar fluorescence profiles. Excitation (ex.) and emission (em.) spectra were taken on an Aviv fluorometer using 390 nm and 485 nm as excitation and emission wavelengths, respectively.

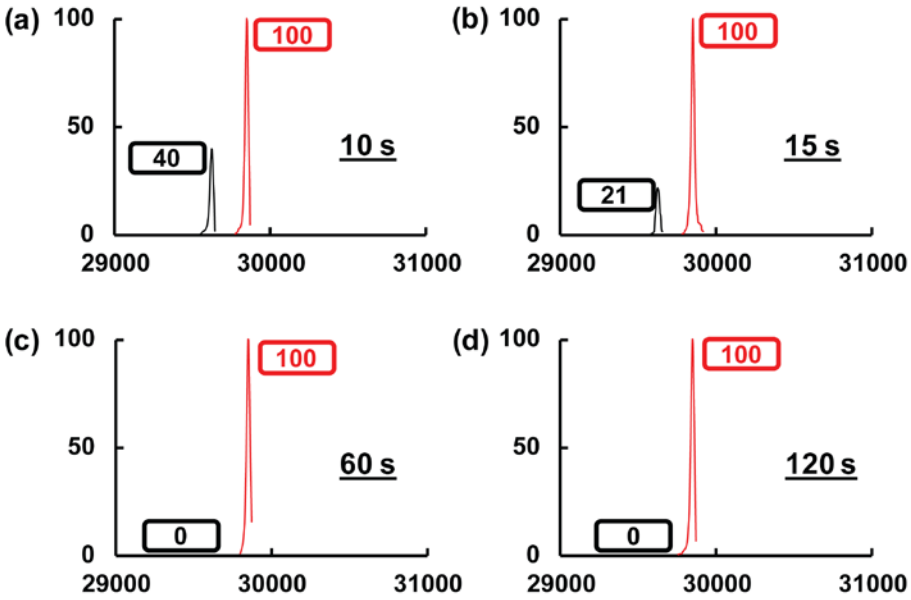

Figure S2. Kinetics of RA-**P1** covalent conjugate formation monitored by LC-ESI-MS. RA (5 µM) was incubated with **P1** (50 µM) at 25 °C in RA buffer (25 mM NaHepes, 100 mM NaCl, pH 7.5). The extent of covalent modification (second step) was monitored by taking samples from the reaction mixture at the indicated time points, quenching the reaction with hydrochloric acid (final pH = 1) and measuring the relative peak intensity by LC-ESI-MS. Relative intensity of RA-**P1** conjugate: red peak and red box; Relative intensity of unmodified apo-RA: black peak and black box. At 10 s, the percentage of modification is 100/(100+40)*100=71.4%. At 15 s, the percentage of modification is 100/(100+21)*100=82.6%.

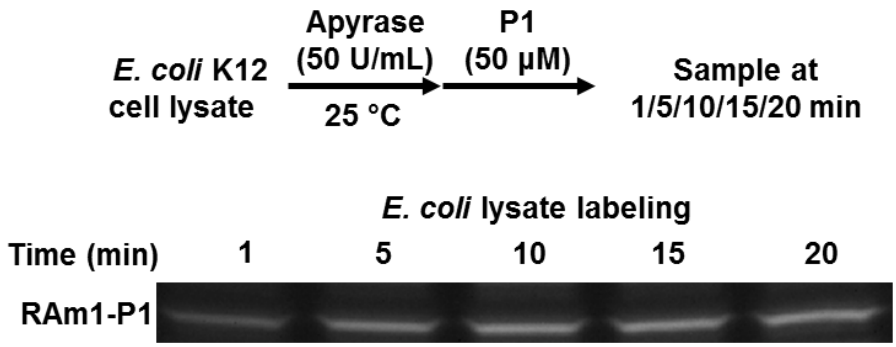

Figure S3. Covalent labeling by **P1** does not significantly change the folding equilibrium or overestimate the amount of folded RAm1; if it did, the intensity of the RAm1-**P1** conjugate fluorescent band would increase with **P1** labeling periods longer than 5-10 min. The total amount of folded and functional RAm1 in *E. coli* was determined by adding 50 μM **P1** to ATP-depleted *E. coli* lysate expressing RAm1. Samples were removed at the indicated time points during the labeling period and subjected to SDS-PAGE. Fluorescence images of the gel was recorded using the Bio-rad Gel Doc Imager employing UV illumination. Complete conjugate formation was observed between 5-10 min. Importantly, the intensity of the conjugate band did not increase after extended incubation periods (15 or 20 min), indicating no shift in the equilibrium between folded and unfolded states of RAm1, consistent with sufficient holdase activity from the ATP depletion step, as demonstrated previously with a battery of experiments in reference 1.

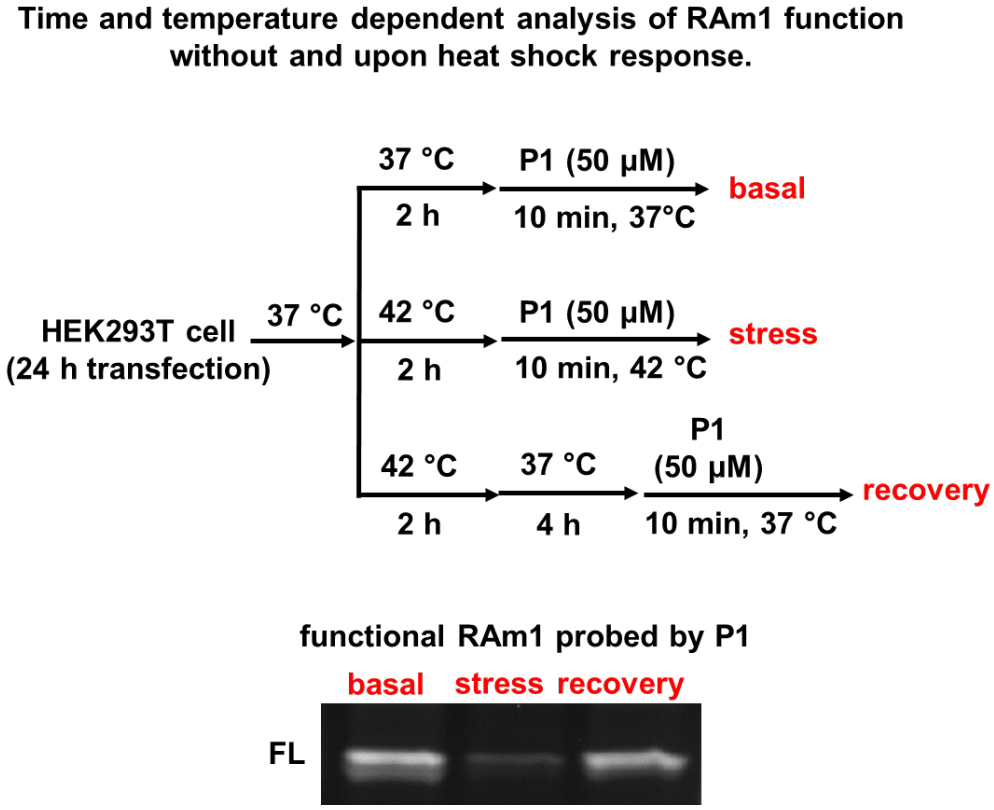

Figure S4. Time- and temperature-dependent analysis of the amount of functional RAm1 in HEK293T cells without and upon a heat shock response, quantified by making the RAm1-**P1** conjugate. The concentration of functional RAm1 at 37 °C vs during the heat shock response (42 °C) and after recovery was probed by adding **P1** and subsequently an SDS-PAGE gel was run and imaged using RAm1-**P1** conjugate fluorescence. Upon heat shock, RAm1 misfolds into a non-functional state (lower panel, middle lane). After an additional 4 h recovery period, cells accomodate the heat stress by turning on the cellular heat shock response, restoring proteostasis network capacity to fold newly synthesized RAm1.

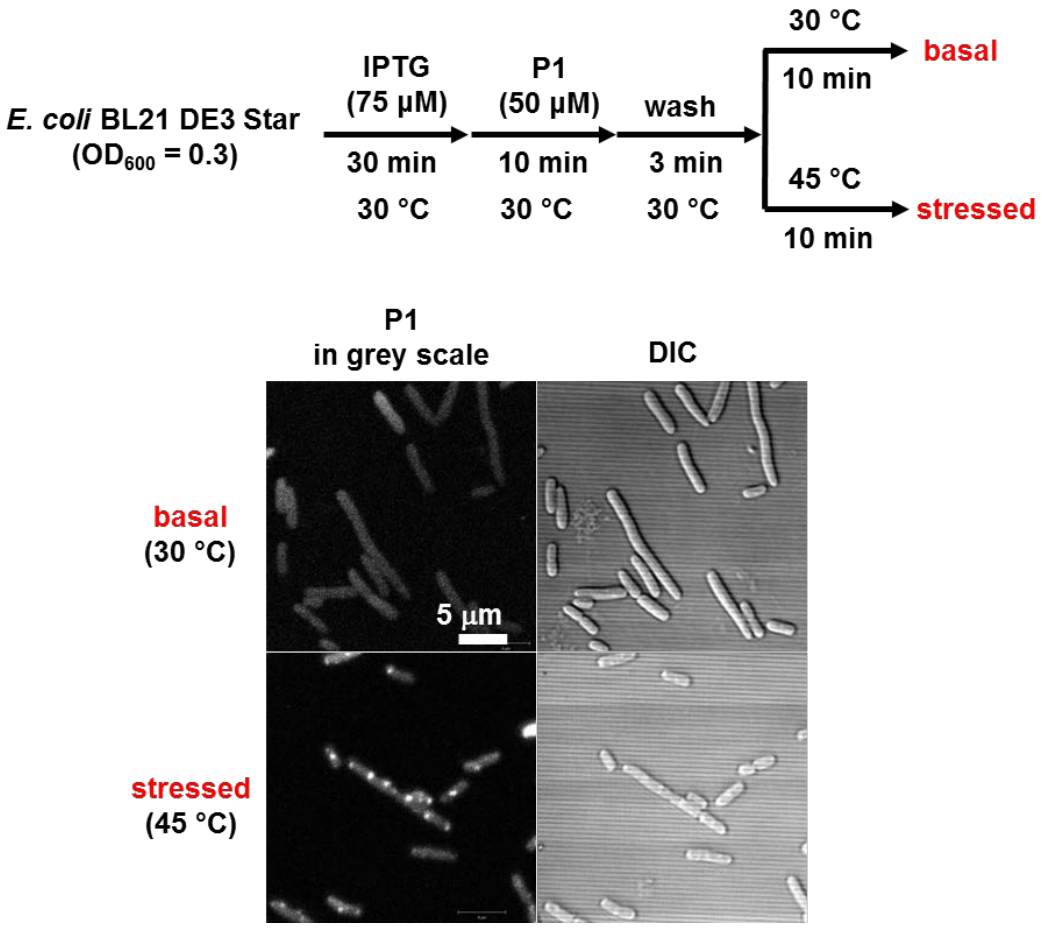

Figure S5. RAm1 is a cellular client-based thermo-labile sensor of proteostasis network capacity in *E. coli* as exemplified by these data in *E. coli* BL21 DE3 Star cells. The pre-formed RAm1-**P1** conjugate formed granular structures after heating at 45 °C for 10 min, serving as a sensor of proteostasis network capacity insufficiency by confocal fluorescence conjugate imaging. Sample preparation and imaging details are described in experimental section in the supporting information. DIC = differential interference contrast.

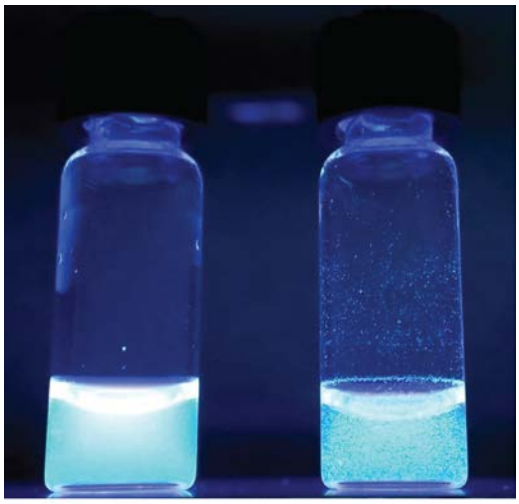

Figure S6. The preformed RAm1-**P1** conjugate forms fluorescent aggregates after heating in RA buffer (defined in Figure S2). Recombinant RAm1 (5 μM) was incubated with **P1** (5 μM) for 12 h in RA buffer. The sample was split equally into two glass vials. One sample was heated to 90 °C for 5 min (right) while the other remained at ambient temperature (left). Samples were photographed under illumination with a hand-held UV lamp.

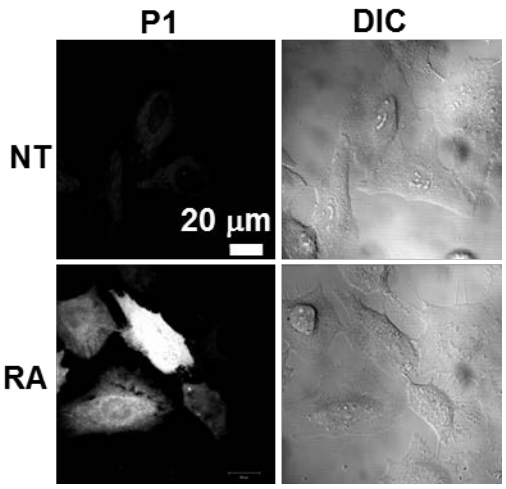

Figure S7. P1 selectively binds to and reacts with RA and exhibits fluorescence only in the HeLa cells transfected with RA. We expressed RA in HeLa cells and then treated the cells with **P1** (10 μM) for 10 min followed by cell washing to remove excess **P1** (for more details see experimental section). Importantly, no off-target fluorescence was observed in the non-transfected (NT) HeLa cells (upper panel). Selective labeling of isolated transfected cells was clear from the observed fluorescence (bottom left panel). Importantly, we also observed unlabeled cells in the transfected samples (lower panel), due to incomplete transfection, which is typical. NT: Non-transfected; DIC: differential interference contrast.

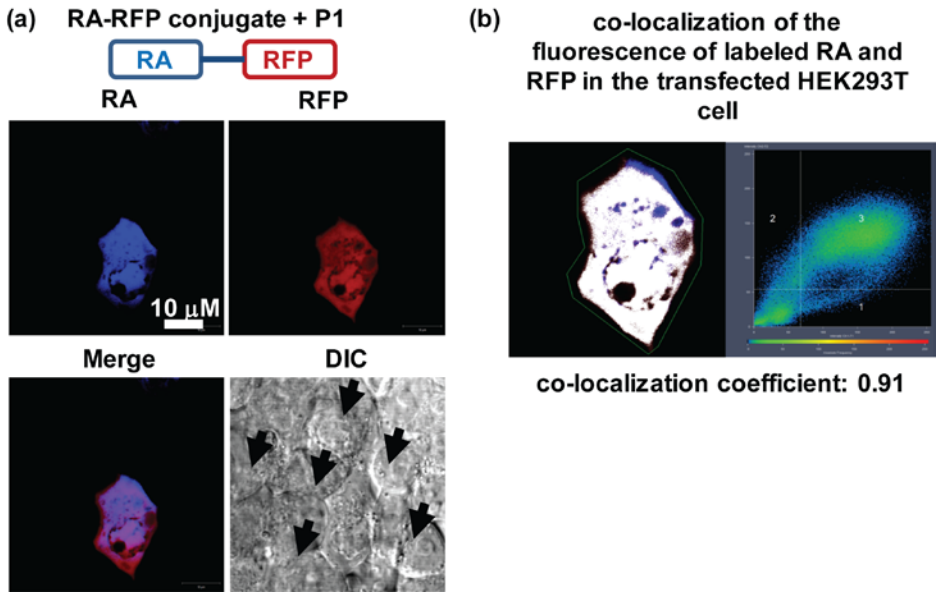

Figure S8. The fluorescence emerging within HEK293T cells originates from **P1** selectively covalently labeling RA. HEK293T cells overexpressing a RA-RFP fusion protein were treated with **P1** (10 μM) for 10 min before confocal images were taken. **P1** fluorescence was visualized using blue fluorescence chanel and RFP fluorescence was visualized using red fluorescence chanel. (a) Co-localization of the **P1** labeling (in blue) and fluorescence of RFP (in red). Only one cell was transfected in the chosen field. Black arrows indicate all the non-transfected cells in the DIC image. (b) The co-localization coefficient is 0.91. DIC: differential interference contrast; Merge: **P1** fluorescence was merged with RFP fluorescence to validate the co-localization.

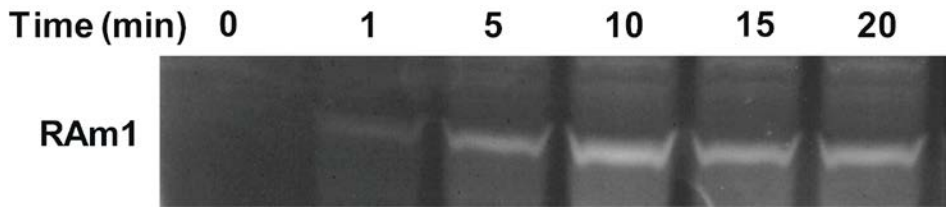

Figure S9. In vivo labeling kinetics of RAm1 by **P1**. HEK293T cells were transiently transfected with RAm1 for 24 h before replacing the media with fresh media containing **P1** (10 μM). Cells were harvested at indicated time points and lysed. Samples were normalized by total protein concentration before loading on an SDS-PAGE gel. Fluorescence image was taken using a Bio-rad Gel Doc Imager employing UV illumination. Ten minutes is sufficient to label the majority, if not all, of RAm1 in live cells, as a saturated RAm1-**P1** fluorescent conjugate signal was observed after 10 min by SDS-PAGE analysis.

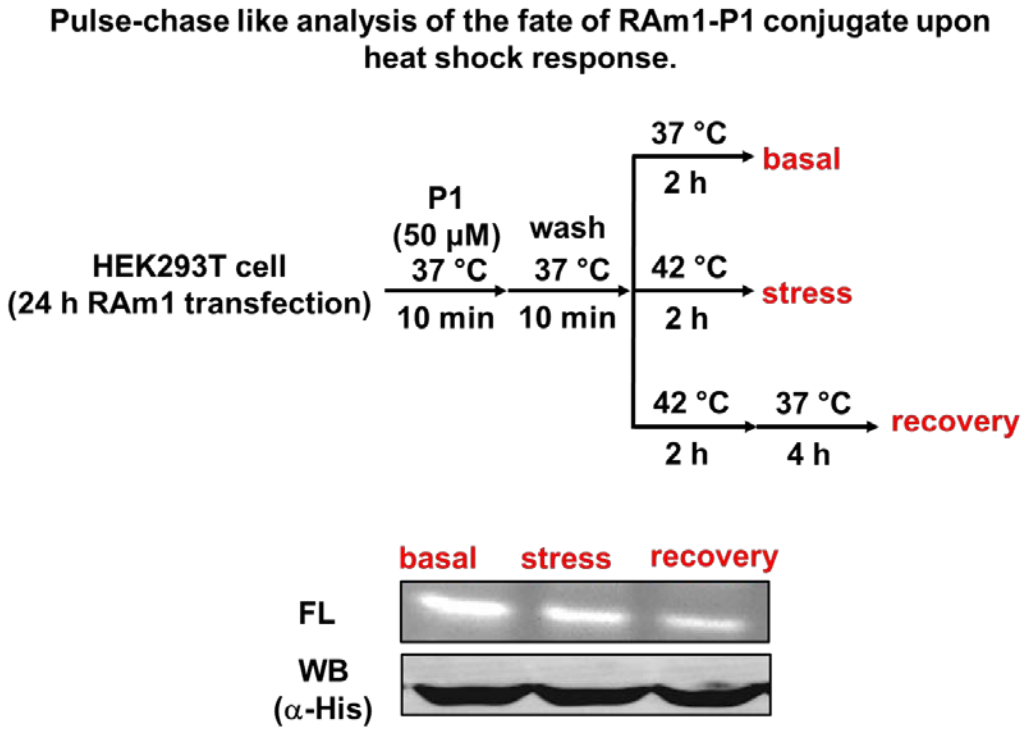

Figure S10. Pulse-chase like analysis of the fate of the RAm1-**P1** conjugate formed during the 10-min **P1** pulse period (no need to attenuate protein translation). The concentration of fluorescent RAm1-**P1** conjugate in HEK293T cells decreases after 2 h of heat stress at 42 °C as shown by SDS-PAGE gel. Proteolytic degradation of the RAm1-**P1** conjugate is more obvious after an additional 4 h recovery period at 37 °C. However, the total amount of RAm1 in the cell, as reflected by SDS-PAGE/immunoblotting (lower panel), does not fluctuate during the heat shock response, likely because the continuous translation of RAm1 balances the degradation of RAm1-**P1** conjugate.

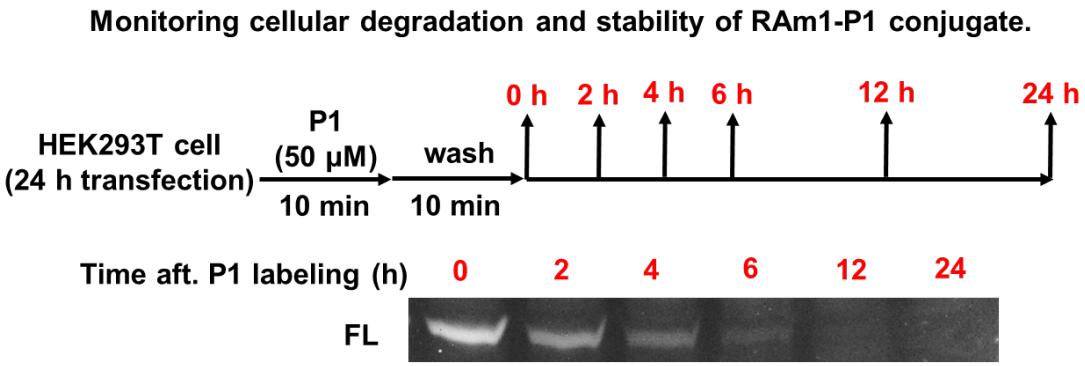

Figure S11. Monitoring cellular degradation of the RAm1-**P1** conjugate formed in the 10-min pulse period (attenuating protein translation is not necessary). Cells were harvested at indicated time points and samples were loaded on an SDS-PAGE gel and the fluorescence image was taken on a Bio-rad Gel Doc Imager using a UV illuminator. After 6-12 h of chase, the RAm1-**P1** conjugate formed during the pulse period is completely degraded.

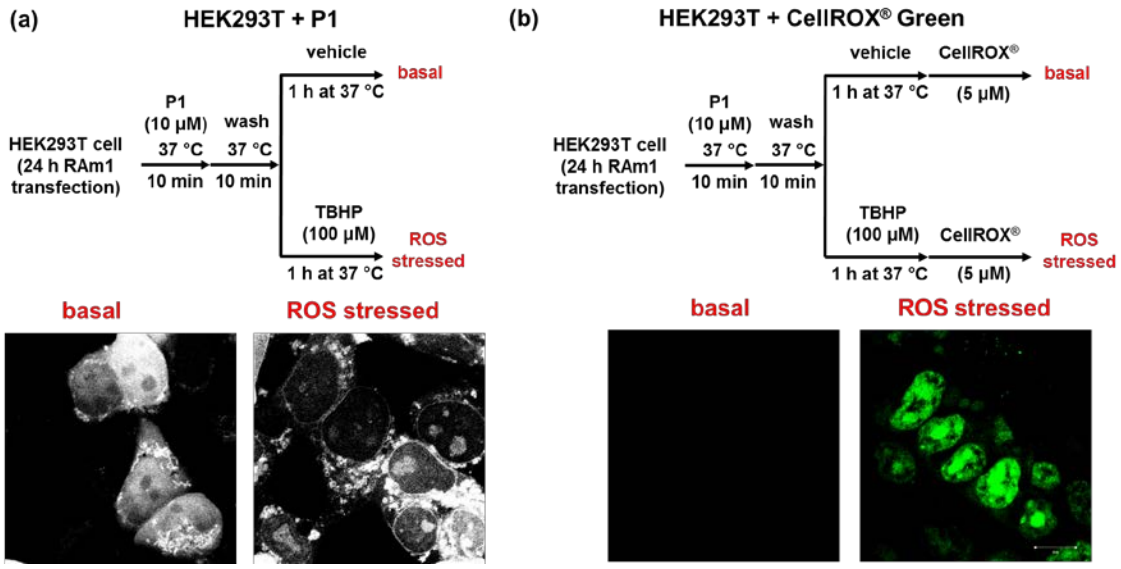

Figure S12. The preformed RAm1-**P1** conjugate can serve as a cellular oxidative stress sensor. Small granular structures were observed in addition to diffuse folded and soluble preformed RAm1-**P1** conjugate in the absence of oxidative stress (Figure S12a, left panel). Upon triggering oxidative stress, the preformed RAm1-**P1** conjugate folding sensor was completely transformed to aggregates. (a) The fluorescent RAm1-**P1** conjugate formed during the pulse period forms large aggregated granular structures under oxidative stress conditions effected by addition of tert-butyl hydroperoxide (TBHP; 100 μM). HEK293T cells were transfected with RAm1 for 24 h. **P1** (10 μM) was added to label RAm1 in cells for 10 min followed by a 10-min wash to remove **P1** from the media. To induce the cellular oxidative stress, TBHP was introduced into the media for 1 h before confocal imaging commenced. (b) Induction of oxidative stress was validated by the addition of the oxidative stress detection probe, CellROX® Green Reagent (5 μM) to both the basal and stressed samples. After 30 min of incubation, samples were washed three times and images were taken by confocal microscopy using the FITC (green) channel. Basal sample without TBHP treatment showed no green fluorescence , whereas the ROS stressed sample showed green fluorescence, which is an indicator for ROS stress.

Experimental Section

(1) Cell labeling and confocal microscopy imaging.

To visualize the fluorescence distribution in *E. coli*, *E. coli* (BL21 DE3 Star strain) cells were transformed with pET29b (+) vectors encoding RA or RAm1. The cells were grown in LB media (1.5 mL cultures) at 30 °C. When the bacteria reached an OD_{600} of 0.3, expression of RA or RAm1 was induced by addition of a low concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG, BioPioneer Inc., San Diego, CA; 75 μM) for 30 min. **P1** (50 μM) was introduced into the media and the bacteria incubated for 10 min. The cultures were centrifuged for 3 min at 3000 g and the media were then replaced with fresh LB media without **P1**. This step removed the unreacted **P1**. One set of samples (RA and RAm1) was subjected to a 45 °C water bath for 10 min (transient heat stress) and the other set of samples (RA and RAm1) was maintained at 30 °C. A 1 μL aliquot of the samples (RA 30 °C, RA 45 °C, RAm1 30 °C, and RAm1 45 °C) was placed onto a chambered coverglass (Thermo Scientific™ Nunc™ Lab-Tek™) and the cells were immobilized with agarose gel, as described previously.[2] The bacteria were then visualized by confocal microscopy using a Zeiss LSM 710 laser scanning confocal microscope (LSCM) attached to a Zeiss Observer Z1 microscope equipped with the infinity corrected optics: 100x oil Plan Apo, 1.4na DIC. The following lasers were used for imaging: 405 nm excitation for detection of **P1**. Appropriate holistic prism based emission was used to detect the signal of each of the dyes respectively.

HEK293T or HeLa cells were grown on chambered coverglasses in DMEM medium and transiently transfected with RA or RAm1 in a pT-Rex vector using X-treme Gene 9 DNA transfection reagent (Roche) with the cells at 60-70% confluency. The

proteins were overexpressed for 24 h. The medium was replaced with DMEM media containing 10 µM **P1** and the cells were incubated for 5 min at 37 °C. The media were then replaced with fresh DMEM media. Two chambers that were transfected with RA or RAm1 were transferred into an incubator at 42 °C for 2 h for thermal stress, while the other two remained at 37 °C. Confocal images were then taken using a Zeiss LSM 710 laser scanning confocal microscope (LSCM). Recovery was performed by shifting heat-stressed samples back to 37 °C for 4 h and then the cells were imaged using the same confocal imaging conditions. Zeiss LSM 710 laser scanning confocal microscope (LSCM) attached to a Zeiss Observer Z1 microscope equipped with the infinity corrected optics: 63x oil Plan Apo, 1.4na DIC. The following lasers were used for imaging: 405 nm excitation for detection of **P1**, and 633 nm excitation for detection of NucRed®. Appropriate holistic prism based emission was used to detect the signal of each of the dyes respectively.

(2) *E. coli* and mammalian lysate preparation for selectivity, solubility and functional analysis.

E. coli strain HMS174 expressing RA (1 mM IPTG induction for 1 h at 30 °C) was used to show the selectivity of **P1** in *E. coli*. Cell lysate was obtained by sonication of the resuspended cells in RA buffer (25 mM NaHepes, 100 mM NaCl, pH 7.5) supplemented with Protease Inhibitor Cocktail (Roche) and apyrase (Sigma, 50 U/mL). The lysates were centrifuged at 16,000 g for 20 min at 4 °C and the supernatant collected as soluble lysates. For mammalian cells, HEK293T cells were transfected with pT-Rex-RA at 60% confluency using X-treme Gene 9 DNA transfection reagent (Roche). After 24 h of expression, the cells were harvested and lysed by sonication. Total protein

concentrations of all soluble lysates were measured using the BCA assay (Thermo Scientific) and normalized to 3 mg/mL. Lysates were treated with **P1** (10 μM) for 20 min before being electrophoresed on an SDS-PAGE gel. Bands were visualized using a UV illuminator.

For the solubility and functional experiments, *E. coli* strain HMS174 expressing RA and RAm1 (50 μM IPTG induction for 30 min at 30 °C) were grown in 4 flasks. Two flasks (RA and RAm1) were incubated in a 45 °C water bath for 10 min while the other two (RA and RAm1) remained at 30 °C. The cells were then harvested and resuspended in RA buffer supplemented with Protease Inhibitor Cocktail (Roche) and apyrase (50U/mL). The lysates were centrifuged at 16,000 g for 30 min at 4 °C and the supernatant collected as soluble lysates. The pellets were resuspended in RA buffer as the insoluble fraction.

(3) Specific activity calculation.

The retro-aldol reaction was performed using 4-hydroxy-4-(6-methoxy-2-naphthyl)-2-butanone as substrate in RA buffer at 25 ºC. Accumulation of product was monitored using a Gemini™ EM Fluorescence Microplate Reader with an excitation wavelength of 330 nm and an emission wavelength of 452 nm. To measure the residual specific activity of RA or RAm1, *E. coli* soluble lysates (50 μL) overexpressing RA or RAm1 were incubated at ambient temperature (25 °C) or heated to 60 °C and samples were collected at the indicated time points (0.5, 1, 1.5, 2 and 3 h). The samples were then cooled down to 25 °C and mixed with RA substrate (500 μM, in excess). The retroaldol activity of RA or RAm1 was reported by the apparent rate derived from the fluorescence signal (ex. 330 nm, em. 352 nm). The specific activity was calculated by normalizing

these rates using the total amount of RA protein in the lysate, as measured by western blot. For easy comparison, we further normalized the specific activity by setting the initial highest specific activity (RAm1, 60 °C, 0 min time point) as 100%.

(4) Measurement of the bimolecular association rate constant of the fluorogenic probe and RA.

The extent of covalent modification (second step) was monitored by taking samples from the reaction mixture at the indicated time points, quenching the reaction by acidification with hydrochloric acid and measuring the relative peak intensity on LC-ESI-MS. The binding kinetics was fitted to a bimolecular binding kinetics model. RA of fixed concentration (5 μM) was mixed with excess and increasing amount of **P1** (25, 37.5, 50, 75 and 100 μM) at 25 °C. Fluorescence (ex. 390 nm, em 485 nm) was recorded using a stopped-flow fluorometer (Aviv, Model 305SF) in real time. The intensity of the fluorescence signal (F_t) was plotted as a function of time to derive the observed rate (k_{obsd}) at different concentrations of **P1** using the equation

$$Ft = (F\infty) \cdot \exp(-k_{obsd} \cdot t),$$

where F_∞ is the saturated fluorescence intensity of RA-**P1**. By plotting k_{obsd} as a function of **P1** concentrations, the bimolecular association rate constant ($k_{bimolecular}$) was derived using pseudo-first-order kinetics under the experiment conditions described above.

(5) Fluorescence electrophoresis gel and western blot analysis.

SDS loading buffer was added to each lysate sample, and the samples were boiled for 5 min. Proteins were resolved by SDS-PAGE on a gradient gel (4-20%). To visualize the RA-**P1** conjugate fluorescence, wet slab gels were scanned using a Bio-RAD Gel Doc Imager with a UV illuminator.

For western blot analysis, the wet slab gels were transferred to a PVDF membrane, which was subsequently blocked with 5% non-fat milk in PBST (PBS containing 0.05% Tween-20) for 1 h at 25 °C. Western blot analysis was performed with a mouse monoclonal α-His antibody (372900, Invitrogen) against His-tagged RA and RAm1 followed by secondary α-mouse (IRDye 800, LI-COR Corp.). The membrane was scanned using a LI-COR Odyssey Imager.

(6) Protein purification of RA and its mutants.

The overexpression and purification follows previously reported protocols.[3] The pET29b (+) vectors encoding RA, RAm1 and RA K210A were transformed into BL21 DE3 Star competent cells (Invitrogen, Carlsbad, CA). When the bacteria reached an OD_{600} of 0.7, protein expression was induced with IPTG (1 mM) for 4 h at 37 ºC. His-tagged RAs were affinity purified using 10 mL of Ni-NTA His•Bind resin (Qiagen, Valencia, CA) in 25 mM HEPES, 100 mM NaCl, 10 mM imidazole, pH 7.5, which was eluted with an imidazole gradient (10 mM to 1 M imidazole over a 100 mL volume). RA-containing fractions were pooled and further purified by size exclusion chromatography (Superdex 75, GE Healthcare) equilibrated in RA buffer (25 mM HEPES, 100 mM NaCl, pH 7.5). The purity of the enzymes was determined to be > 98% by LC-ESI-MS and electrophoresis analyses.

(7) Plasmids and vectors constructions.

a) *Construction of **RA** and **RAm1** vector for mammalian cell expression.* The RA and RAm1 gene was subcloned from the previously reported pET29b (+) vectors[1] to the pENTR1A vector. Subsequently, the RA1 gene was shuttled from pENTR1A into pT-

Rex-DEST30 vector using LR clonase II (Invitrogen, Inc.) recombination to generate the pT-Rex-RA1 vector for expression in mammalian cells.

b) *Construction of **RA:RFP** vector.* pRA:RFP vector was cloned from the previously reported vector pRA:RFP:NLS[1] by deleting the three repeats of the NLS sequence using the Stratagene Quikchange method. The primer used was

CGCCGAGGCCCGCCACTAACTGATCATAATCAGCC.

(8) General materials information and chromatographic methods.

All reagents and anhydrous solvents of commercial grade were used as received unless stated otherwise. All reactions were carried out under Argon gas unless stated otherwise. **P1** was purchased from Invitrogen and used as it is. Purity was confirmed by LCMS. LC/MS analysis was performed on an Agilent G1956 single quadrapole mass spectrometer coupled with an Agilent 1100 LC stack. Solvents used are H_2O/0.1% formic acid and acetonitrile/0.1% formic acid.

References:

(1) Liu, Y.; Tan, Y. L.; Zhang, X.; Bhabha, G.; Ekiert, D. C.; Genereux, J. C.; Cho, Y.; Kipnis, Y.; Bjelic, S.; Baker, D.; Kelly, J. W. *Proc Natl Acad Sci U S A* **2014**, *111*, 4449.

(2) Skinner, S. O.; Sepulveda, L. A.; Xu, H.; Golding, I. *Nat. Protoc.* **2013**, *8*, 1100.

(3) Liu, Y.; Zhang, X.; Tan, Y. L.; Bhabha, G.; Ekiert, D. C.; Kipnis, Y.; Bjelic, S.; Baker, D.; Kelly, J. W. *J. Am. Chem. Soc.* **2014**, *136*, 13102.