Supporting Online Material for

"Regulated Fast Nucleocytoplasmic Shuttling Observed

by Reversible Protein Highlighting"

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Materials and Methods

cDNA cloning and gene construction. Total RNA isolation, cDNA synthesis, and the production of a directional cDNA library using pRSET-FastBac and the *E.coli* strain JM109 (DE3) were performed as previously described (*S1*). Colonies were screened for fluorescence using a UV-illuminator (365 nm) or our homemade fluorescence analyzing system (*S2*). Mammalian expression was performed after cloning fragments into the pcDNA3 vector (Invitrogen).

Protein expression and in vitro spectroscopy. Proteins were expressed in *E.coli*, purified, and spectroscopically characterized according to established methods (*S1*). The quantum yields of the fluorescence, photoactivation, and photobleaching were calculated as previously described (*S3*). Absorbance spectra were determined on a Hitachi U-3310 spectrophotometer. Fluorescence measurements were carried out on a Hitachi F-2500 fluorescence spectrophotometer.

Cell culture. Cells grown on a 35-mm glass-bottomed dish were imaged two to five days after cDNA transfection with lipofectin (Invitrogen, Gaithersburg, MD).

Analysis of the photochromic kinetics of Dronpa. HeLa cells expressing Dronpa were fixed in 4% paraformaldehyde for 10 minutes and imaged on a microscope (Olympus IX70) with a standard 75-W xenon lamp and a 40x objective lens (UApo/340, N.A. = 1.35). The movement of the interference filters for both excitation and emission was automated using Lambda 10-2 hardware (Sutter Instruments, Novato, CA). The filter-switching time was set to 50 ms. Emitted fluorescence was captured by a cooled CCD camera (Cool $SNAP_{HQ}$, Roper Scientific, Tucson, AZ). The exposure time was 100 ms. The entire system was controlled using MetaFluor 4.5 software (Universal Imaging, Media, PA).

Writing, reading, and erasing letters on a coverslip. Purified Dronpa protein in a solution containing 50 mM N-hydroxysuccinimide and 200 mM N-ethyl-N'-(dimethylaminopropyl) carbodiimide were immobilized onto a poly-L-lysine-coated coverslip. The sample was imaged using a confocal microscope (Fluoview FV500, Olympus) equipped with acousto-optic tunable filters, a 40x objective lens (UPlan Fl, N.A.=1.30) and two laser lines: a 405-nm laser diode and a 488-nm argon laser. The photoactivation patterns for the letter drawing were designed using FV500 software.

Imaging of the nucleocytoplasmic shuttling of ERK1-Dronpa and importin-b-Dronpa. Intermediately bright cells were chosen for imaging experiments. COS7 cells expressing ERK1-Dronpa or importin-β-Dronpa were visualized using a confocal microscope equipped with a 60x objective lens (Plan Apo, N.A.=1.40) and two scanning units (Fluoview FV1000, Olympus). The system operates a laser diode (405 nm) and an argon laser (488 nm) independently, enabling the simultaneous execution of photoactivation or photobleaching together with fluorescence observation.

Measurement of light power density. The power density of the excitation light at the level of the cells was measured using a homemade power meter (*S1*). The light exiting an objective lens was collected on the front surface of the meter, and the total power of the light (watts) was measured by thermal detection. The power density was obtained by dividing the total power by the area of a scanned region or an illumination spot for laser-scanning confocal and wide-field microscopy, respectively.

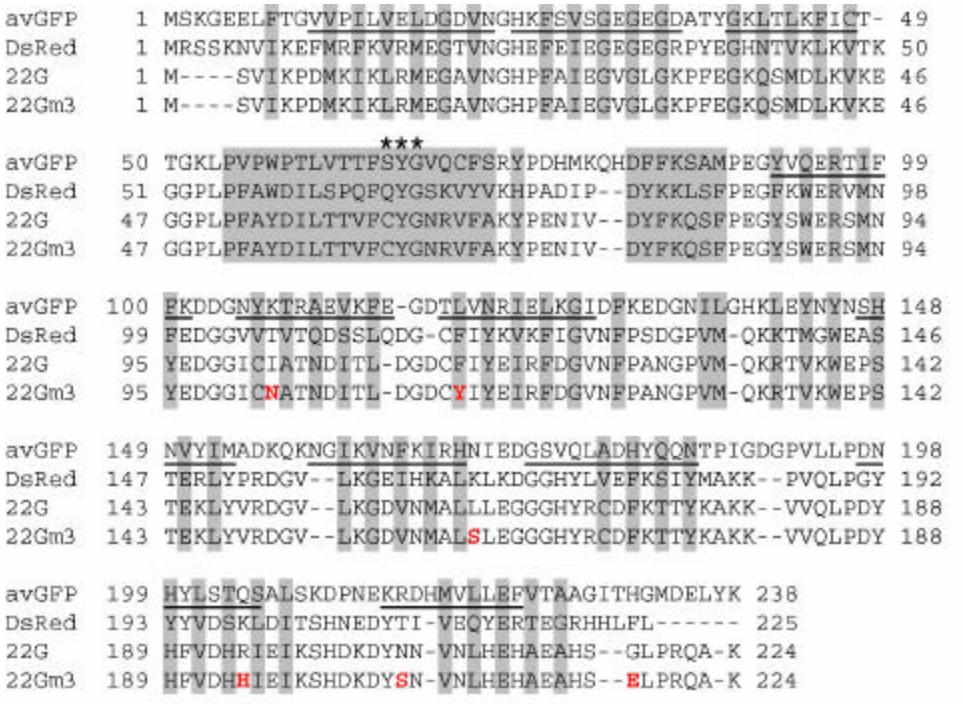

Fig. S1

Amino-acid sequence (single-letter code) alignment of 22G and 22Gm3 (Dronpa) with native GFP (*Aequorea victoria* GFP) (*S4*) and DsRed (*S5*). In the GFP sequence, the β-sheet-forming regions are underlined. Residues whose side chains form the interior of the β-can (*S4*) are shaded. Residues responsible for chromophore synthesis are indicated by asterisks. Amino acids substituted to create 22Gm3 (Dronpa) are indicated in red. The DDBJ accession number for Dronpa (22Gm3) is AB180726.

Table S1. Comparison between PA-GFP and Dronpa.

	$\varepsilon^{?}$	$\Phi_{FL}{}^{\dagger}$	$\Phi_{PA}{}^{\ddagger}$	$\Phi_{PB}{}^{\|\|}$	Reversibility	Ref.
PA-GFP	17,400	0.79	NS[§]	---	irreversible	S6
Dronpa	95,000	0.85	0.37	0.00032	reversible	---

[*]ε, extinction coefficient (in $M^{-1}\bullet cm^{-1}$) in the bright state; [†]Φ_{FL}, the quantum yield for fluorescence; [‡]Φ_{PA}, the quantum yield for photoactivation; [§]NS, not stated in the reference; [||]Φ_{PB}, the quantum yield for photobleaching.

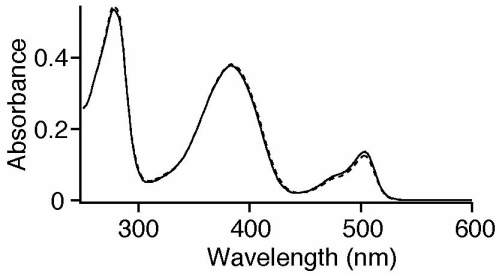

Fig. S2

The absorbance spectrum of acid-quenched Dronpa (pH = 4.0) was measured (solid line). The sample was irradiated at 400 ± 7.5 nm for six minutes, and then its absorbance was measured again (broken line). There was no change in the absorbance spectrum. The irradiation procedure completely converted the photobleached Dronpa to its original emissive state (Fig. 1B).

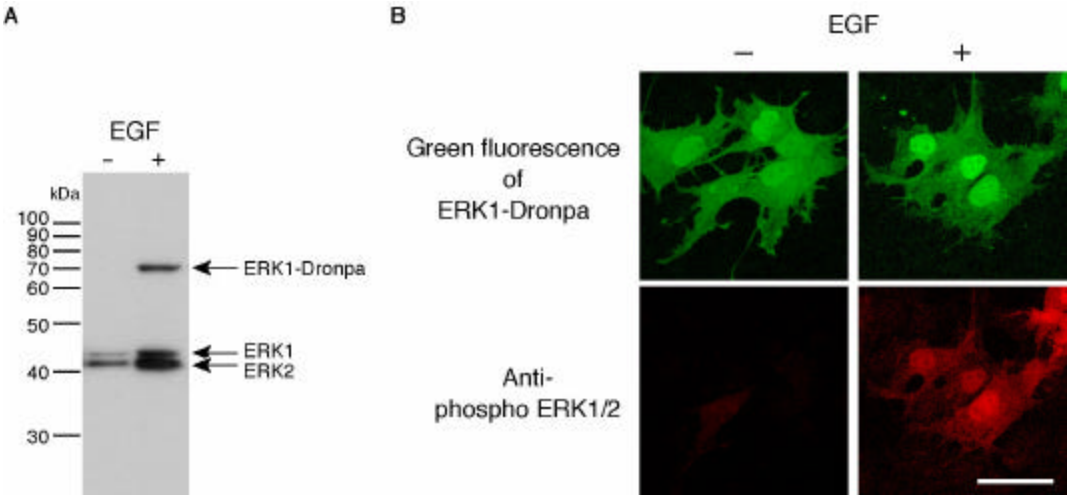

Fig. S3

EGF-dependent phosphorylation and nuclear accumulation of ERK1-Dronpa in COS7 cells. A monoclonal antibody to activated MAP kinase activated (diphosphorylated ERK1/2) (Sigma #8159) was used. (**A**) Western blotting revealed that ERK1-Dronpa as well as the endogenous ERK1/2 were phosphorylated after incubation with 100 ng/ml EGF for 10 minutes. (**B**) Immunocytochemistry revealed that the phosphorylation of ERK1-Dronpa (and ERK1/2/) was significantly concentrated in the nucleus after incubation with 100 ng/ml EGF for 10 minutes. The distribution of Dronpa's green fluorescence was well correlated with that of the immunofluorescence signal in transfected cells. Scale bar, 40 μm.

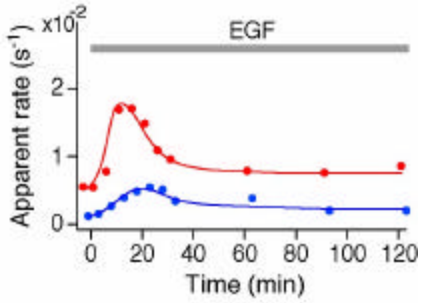

Fig. S4

A typical time course of ERK1-Dronpa nuclear influx (red) and efflux (blue) rates in a COS7 cell stimulated with 10 ng/ml EGF.

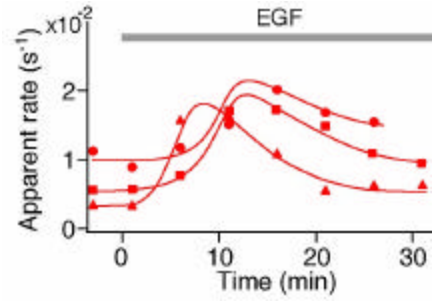

Fig. S5

Time courses of the nuclear influx (red) rates of ERK1-Dronpa during stimulation with 10 ng/ml EGF obtained from different three COS7 cells (circles, squares, and triangles).

Additional References

S1. R. Ando, H. Hama, M. Yamamoto-Hino, H. Mizuno, A. Miyawaki, *Proc. Natl. Acad. Sci. USA* **99,** 12651 (2002).

S2. A. Sawano, A. Miyawaki, *Nucleic Acids Res.* **28,** e78 (2000).

S3. S. R. Adams, J. P. Y. Kao, G. Grynkiewicz, A. Minta, R. Y. Tsien, *J. Am. Chem. Soc*. **110,** 3212 (1988).

S4. R. Y. Tsien, *Annu. Rev. Biochem.* **67,** 509 (1998).

S5. M. V. Matz *et al., Nat. Biotechnol.* **17,** 969 (1999).

S6. G. H. Patterson, J. Lippincott-Schwartz, *Science* **297,** 1873 (2002).

Movie S1. This movie shows the nuclear import and export of ERK1-Dronpa in COS7 cells stimulated with 100 ng/ml EGF. Images of two representative monitoring experiments (t = 11 and 13 min) and the timetable are shown in Fig. 2.