Supporting Information

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SI Materials and Methods

Light Miscroscopy. Images were collected with an Olympus 81× inverted microscope, equipped with bright field and fluorescence optics, using SlideBook v 4.1 software (3I Corp.). Images were collected with 20× or 63× objectives and an ORCA-ER camera. The SlideBook images were cropped using PhotoShop and annotated using Canvas. At each time point, 8–15 random fields of cells were chosen under bright field illumination and photographed both with the transmitted light and the fluorescence channels, using uniform exposure times and gain settings. The numbers of transfected (fluorescent) and nontransfected cells in each field were determined and the average from each time point was used to generate a cell survival curve. To ascertain ER expression of the fusion proteins, GRP94-mCherry was cotransfected with either an ER-targeted GFP (44) or with a cytosolic GFP construct.

Quantitation of Cell Fluorescence. for each time point, 8–15 fields were photographed under brightfield illumination at 100 msec each, followed by a 500 msec exposure under the GFP filter. Automatic brightness and contrast settings were overridden to obtain the entire camera range. Files were exported in TIFF format into TINA2.10g software, boxes were drawn around 10–21 individual cells or equivalent areas without cells for background values, and the net total pixel intensity of cells was averaged.

Fluorescence Photobleaching Analysis. Live cells were imaged on a 37 °C environmentally controlled chamber of a confocal microscope system (Duoscan; Carl Zeiss Inc.) with a 63×/1.4 NA oil objective and a 489-nm 100 mW diode laser with a 500- to 550-nm bandpass filter for GFP. For FRAP experiments, regions of interest were photobleached at full laser power (489 nm line) and fluorescence recovery was monitored by scanning the whole cell at low laser power. No photobleaching of the cell or adjacent cells during fluorescence recovery was observed. Composite figures were prepared using Photoshop CS2 and Illustrator CS software (Adobe). Diffusion (D) measurements were calculated as described previously (45, 46) and plotted with Prism 4.0c. Statistical significance was assessed using Student's t test.

ELISA Measurement of IGF-II Secretion. High-capacity (96 well) polystyrene plates (Costar) were coated with capture antibody—anti-mouse IGF-II monoclonal antibody 122404 (R&D Systems). After blocking, the antibody-coated plates were incubated for 2 h at room temperature with media of cells that had been incubated without serum for 24 h. Bound IGF was detected with biotin-labeled goat anti-mouse IGF-II, followed by Streptavidin-HRP. After addition of the ABTS substrate (Roche), binding was quantified by absorbance at 415 nm using a Synergy HT plate reader (BIO-TEK).

Immunoprecipitation. Cells expressing the various GRP94 fusion proteins were lysed and immunoprecipitated as described in ref. 15.

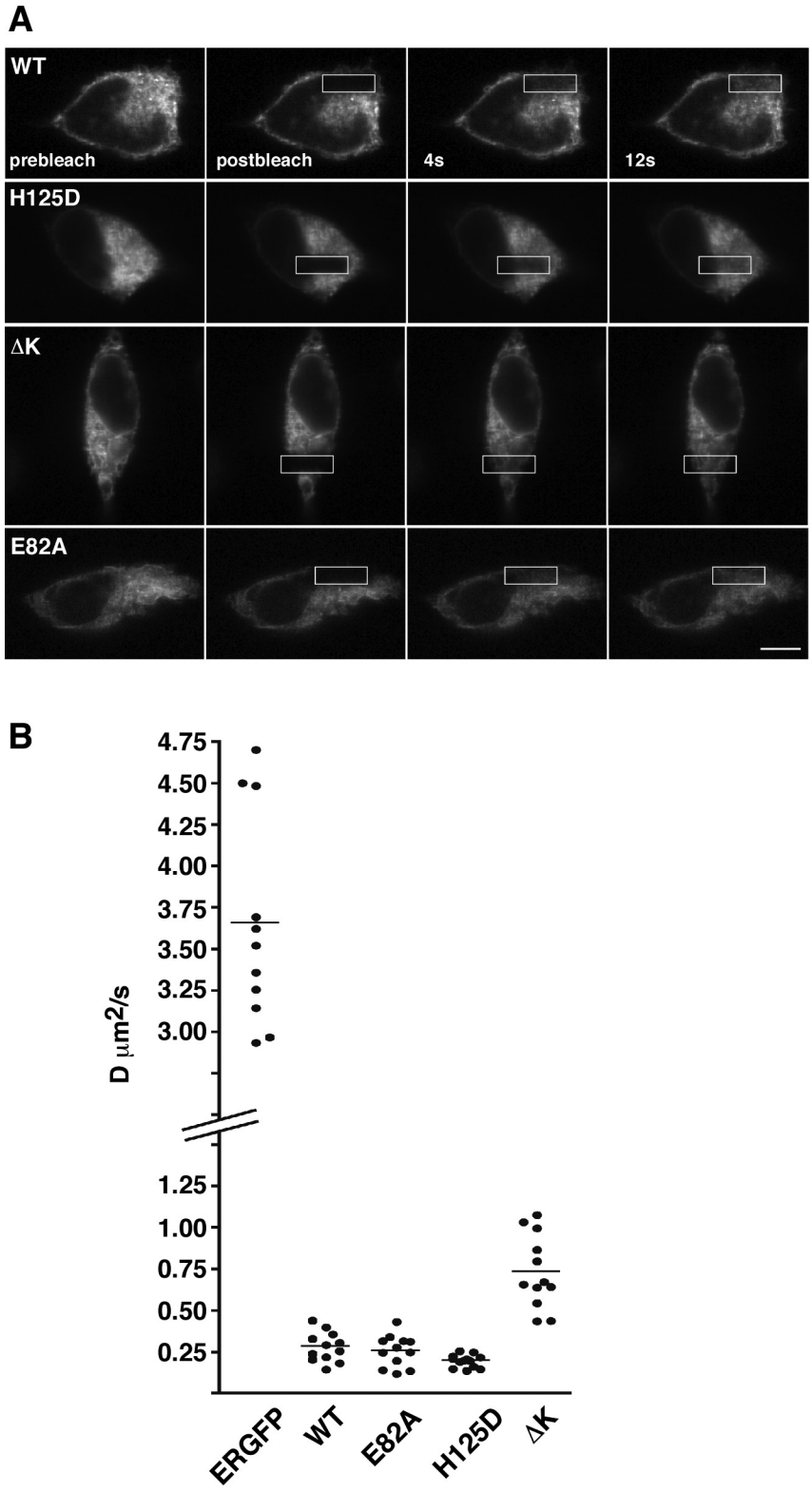

Fig. S1. Mobility of GRP94 mutants. *Grp94*$^{-/-}$ cells expressing either wild-type fusion protein, the ΔK fusion protein, or the H125D or E82A mutants were subjected to photobleaching and the recovery of fluorescence was measured in individual cells. Data shown are from 1 representative experiment. (*A*) Time course of recovery after photobleaching in representative cells. (Scale bar, 10 μm.) (*B*) Quantitative analysis of the diffusional mobility of each version of GRP94.

Table S1. Expression levels of GRP94 mutants

Mutant	Average fluorescence/cell	Relative expression
Wild type	113753	1.00
ΔK	178789	1.57
H125D	151704	1.33
E82A	157052	1.38
D128N	147509	1.30
F396Q	138058	1.21
R427A	130458	1.15
Q431A	129458	1.14

The expression level of each GRP94 construct was quantified by measuring the total fluorescence per cell. The numbers were calculated as the sum of all intensities of fluorescent pixels from each cell, in arbitrary units, divided by the 50 cells that were sampled for each construct. All cells were imaged with the same camera and exposure conditions.