A Novel Destabilizing Domain Based on a Small Molecule-Dependent Fluorophore

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Supporting Information

Table of Contents

Experimental Procedures

UnaG FDD Library Generation: Diversity in the UnaG sequence was generated using error-prone PCR as previously described.[1] Primers for mutagenic PCR were designed to anneal upstream and downstream of the wild type *UnaG* gene, and for cloning the mutagenesis products into a pBMN retroviral expression vector encoding a blasticidin S resistance gene behind an internal ribosomal entry site (iBlast). The following set of conditions were used to generate diversity in the library: condition set #1 utilized the nonnatural nucleotides 8-oxo-dGTP and dPTP to encourage nucleotide misincorporation. Condition set #2 utilized 4 ng template, 0.5 μM of each oligonucleotide primer, 5 units of Taq polymerase, 5 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.4 mM dNTPs, and an excess 0.2 mM of dATP and dCTP. Condition set #3 was identical to #2, but dGTP and dTTP were used in excess instead of dATP and dCTP. The combined library of inserts was cloned into the pBMN-iBlast at the C-terminus of mCherry for expression of mCherry-UnaG fusion proteins. A library of 2×10^5 members was obtained.

Library Screening and Validation: Screening was performed as described in the text. Upon completion of the fourth sort, the cells were allowed to recover in media, and genomic DNA was obtained using QIAGEN DNeasy kit. To isolate individual UnaG mutants from the pool of genomic DNA, the UnaG gene was PCR-amplified using primers that annealed to regions just outside the 5' and 3' end of the gene. PCR products were then subcloned behind mCherry into the pBMN iBlast plasmid and transformed into E. coli Top10 cells. Sixty randomly chosen clones were further characterized in mammalian cells. The plasmid DNA was isolated from bacteria and stably transduced into NIH 3T3 cells using the retroviral expression system described below. The cells were treated with 10 μM bilirubin (BR) (1:2000 dilution of 20 mM stock in DMSO) in media supplemented with charcoal-stripped fetal bovine serum (Invitrogen),[2] or cultured in the absence of BR, which was accomplished by culturing cells in charcoal-stripped media supplemented with bovine serum albumin (BSA, Sigma-Aldrich, 20 mg mL^{-1}). Only the cells that displayed low mCherry/UnaG fluorescence in the absence of BR and high mCherry/UnaG expression in the presence of BR were sequenced. Sequence information is provided in Table S1.

Cell Culture and Viral Transduction: NIH 3T3 cells were cultured in growth media (DMEM supplemented with 10% heat-inactivated donor bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin) (Invitrogen) at 37 °C and 5% CO_2. All stable cell lines were created using the Phoenix ecotropic packaging cell line that was transfected using a standard TransIT-LT1 (Mirus) protocol. Viral supernatants were harvested 48 hr post-transfection, filtered, and supplemented with 4 μg/ml polybrene. NIH 3T3 cells were incubated with viral supernatant for 4 hr at 37 °C. Cells were cultured in growth media for at least 48 hr to allow for viral integration and then assayed as described. For fluorescence decay kinetics experiments, bilirubin (BR) was removed from the cells by washing cells with charcoal-stripped media "conditioned" with bovine serum albumin (BSA; 20 mg mL^{-1}). Cells were then incubated in the conditioned media for the indicated times.

Flow cytometry: Twenty-four hours prior to analysis, transduced NIH3T3 cells were plated and treated with BR as described. Cells were trypsinized, resuspended in growth media, and analyzed at the Stanford Shared FACS Facility. 10,000 events were recorded for every sample.

Microscopy: NIH-3T3 cells expressing fluorescent proteins were imaged with a ×40 objective on a Zeiss Axioskop 2 epifluorescence microscope equipped with a QICAM FAST 1394 digital CCD camera. For nuclear staining, cells were incubated with 1 μg mL^{-1} Hoechst 33342 for 15 min before imaging.

Supplementary Figures

Table S1. Representative sample of BR-sensitive UnaG mutants isolated from the library screen. NIH 3T3 cells stably expressing the indicated mCherry-UnaG fusion proteins derived from error-prone PCR were treated with or without 20 μM bilirubin (BR) for 24 h, and UnaG and mCherry fluorescence was monitored by analytical flow cytometry. WT = wild-type UnaG.

Clone	no BR UnaG	20 μM BR UnaG	no BR Cherry	20 μM BR Cherry	number of mutations	amino acid changes
untransfected 3T3 cells	943	1185	459	406	-	-
#15	864	3615	558	1488	2	A36V, R136G
#17	818	6234	807	3531	1	V98A
#20	1107	5883	756	3166	2	D13G, G126R
#25	1218	14182	1430	8041	1	K118I
#46	1206	11712	1150	6587	1	S31G
#57	1138	7763	1091	4244	1	V89A
#59	1003	5684	825	3117	1	E107G
WT	4188	25858	6185	14255	-	-

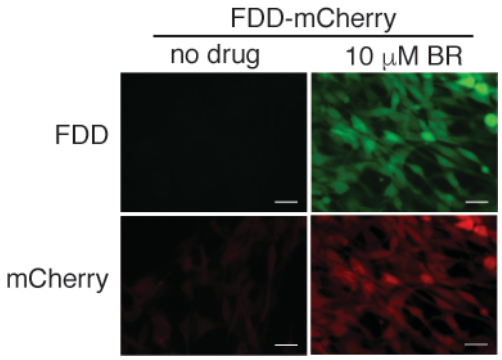

Figure S1. Fluorescence microscopy of NIH 3T3 cells stably expressing FDD-mCherry. Cells were treated with or without 10 μM BR for 24 h then analyzed using epifluoresnce microscopy. Scale bars, 20 μM.

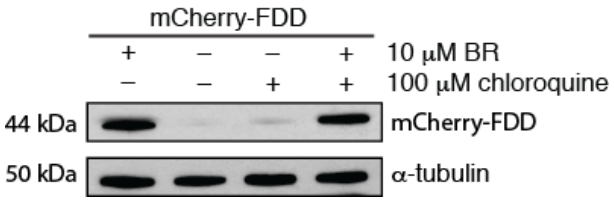

Figure S2. Ligand washout in the presence of chloroquine. NIH 3T3 cells stably expressing the mCherry-FDD fusion were treated with 10 μM BR for 24 h, at which point cells were washed with media to remove ligand and exposed to 100 μM chloroquine for 8 h, then cell lysates were prepared and immunoblotted with antibodies against hemagglutinin antigen (HA; the tag was inserted at the N-terminus of mCherry). α-tubulin served as the loading control.

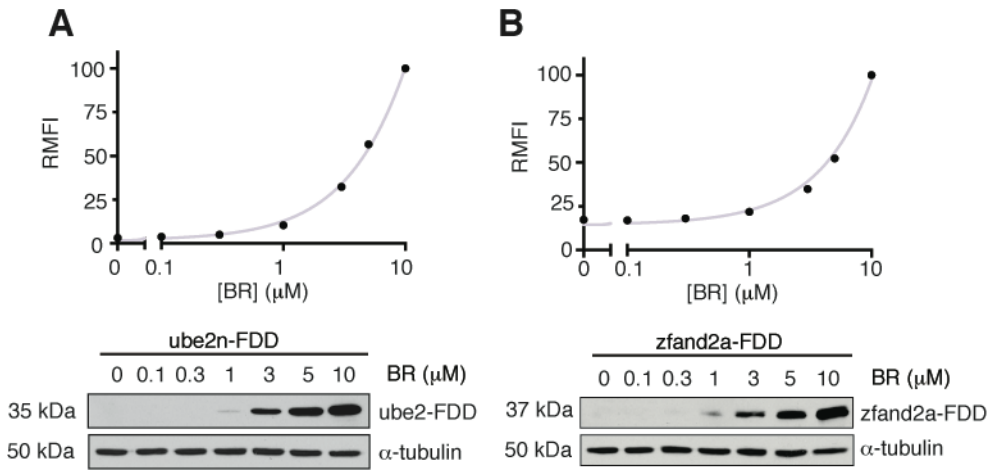

Figure S3. Tunable expression of uben2n-FDD and zfand2a-FDD. A) NIH 3T3 cells stably expressing ube2n-FDD were treated with the indicated concentrations of bilirubin (BR) for 18 h, then analyzed by flow cytometry and immunoblotted using antibodies against HA (tag was inserted at the N-terminus of ube2). B) NIH 3T3 cells stably expressing zfand2a-FDD were treated with the indicated concentrations of bilirubin (BR) for 18 h, then analyzed by flow cytometry and immunoblotted using antibodies against HA (tag was inserted between zfand2a and the FDD). RMFI = relative mean fluorescence intensity.

References

[1] L. A. Banaszynski, L.-C. Chen, L. A. Maynard-Smith, A. G. L. Ooi, T. J. Wandless, *Cell* **2006**, *126*, 995–1004.

[2] Charcoal-stripped serum was implemented because bilirubin is a heme metabolite naturally present in serum commonly used for cell culture.