† **Electronic Supplementary Information (ESI)**

moxDendra2: an inert photoswitchable protein for oxidizing environments

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Experimental procedures

Spectral and photochemical characterization of Dendra2 and moxDendra2. For bacterial expression of the Dendra2 and moxDendra2 nucleotide sequences encoding these proteins were subcloned into a pBAD/His-D vector using *Bgl*II and *Eco*RI restriction sites. Proteins with 6x polyhistidine tags on the N-terminus were expressed in TOP10 bacterial cells. The bacterial cells were grown in RM medium supplemented with ampicillin for 6-8 h followed by an induction of protein expression by adding of 0.002% arabinose. The proteins were purified using a Ni-NTA agarose (Qiagen, Germantown, MD).

For absorbance measurements, a Hitachi U-2000 spectrophotometer was used. Fluorescence was measured by a FluoroMax-3 spectrofluorometer (Horiba-Jobin Yvon, Edison, NJ). A photoconversion of purified proteins was performed with a 405/20 nm custom assembled LED array. Proteins were diluted with PBS to 5 μM and placed on ice in transparent micro tubes with following illumination at 10 mW cm^{-2} for 30 min.

To determine molar extinction coefficients of the green chromophores, we relied on measuring of mature chromophore concentrations. Purified proteins were alkali-denatured and extinction coefficients was calculated according to the known extinction coefficient (44,000 M^{-1} cm^{-1}) of the synthetic compound of the tyrosine-containing GFP-like chromophores at 447 nm in 1 M NaOH[1]. Based on the absorbance of the native and denatured proteins, molar extinction coefficients for the native states were calculated. Molar extinction coefficients of the red chromophores were estimated by comparing the absorption bands corresponding to the pre-photoconverted green species and the absorption band of the red species from photoconverted samples.

Fluorescence quantum yield of the green and red chromophores of moxDendra2 were measured relative to Dendra2[2], according to Williams's method.[3]

To evaluate photostability of proteins, the TOP10 bacterial cells containing target proteins in pBAD/His-D plasmid were grown on LB/ampicillin Petri dishes supplemented with 0.004% arabinose, overnight at 37°C. Bacteria were photobleached using an Olympus IX81 inverted epifluorescence microscope equipped with a 200 W Me-Ha arc lamp (Lumen220Pro, Prior), 60x 1.35 NA oil-immersion objective lens (UPlanSApo, Olympus, Waltham, MA) and 480/40 nm exciter for green form and 540/20 nm exciter for red form (Chroma Technology Corporation, Bellows Falls, VT). An intensity of photobleaching light was 4.4 mW cm^{-2} measured at the back focal plane of objective lens. Fluorescence of green and red form were detected using 480/40 nm exciter and 535/40 nm emitter and 540/20 nm exciter and 575/30 nm emitter sets respectively (Chroma). For measurement of photoconversion half-time 390/40 nm exciter was used. An intensity of photoconversion light was 1.6 mW cm^{-2} measured at the back focal plane of a 60x 1.35 NA oil-immersion objective lens (Olympus).

To construct periplasm targeting versions of Dendra2 and moxDendra2, the fluorescent proteins were fused to the co-translational periplasm targeting maltose binding protein signal peptide.[4, 5] BL21(DE3) bacterial cell (New England Biolabs, Ipswich, MA) cultures were transformed with the constructs. Antibiotic selection was maintained on plates and in cultures using 100 µg/mL ampicillin. 24 h after seeding cells on LB/Agar medium with antibiotics, IPTG was added underneath the agar to a final concentration ~1 mM (20 µL of 1 M IPTG stock). Bacterial cells were imaged after 48 h of expression induction using the Olympus microscope.

Mammalian Plasmids. Engineering of moxEos3.2. mEos3.2 was synthesized by GenScript (Piscataway, NJ) using the high expression human optimized codons described by Haas et al.[6]

Human high expression optimized coding sequence

ATGGTGAGCGCCATCAAGCCCGACATGAAGATCAAGCTGCGCATGGAGGGCAACGTGAAC
GGCCACCACTTCGTGATCGACGGCGACGGCACCGGCAAGCCCTTCGAGGGCAAGCAGAG
CATGGACCTGGAGGTGAAGGAGGGCGGCCCCCTGCCCTTCGCCTTCGACATCCTGACCA
CCGCCTTCCACTACGGCAACCGCGTGTTCGCCAAGTATCCCGACAACATCCAGGACTATTT
CAAGCAGAGCTTCCCCAAGGGCTATAGCTGGGAGCGCAGCCTGACCTTCGAGGACGGCG
GCATCTGCAACGCCCGCAACGACATCACCATGGAGGGCGACACCTTCTATAACAAGGTGC
GCTTCTATGGCACCAACTTCCCCGCCAACGGCCCCGTGATGCAGAAGAAGACCCTGAAGT
GGGAGCCCAGCACCGAGAAGATGTACGTGCGCGACGGCGTGCTGACCGGCGACATCGAG
ATGGCCCTGCTGCTGGAGGGCAACGCCCACTACCGCTGCGACTTCCGCACCACCTACAAG
GCCAAGGAGAAGGGCGTGAAGCTGCCCGGCGCCCACTTCGTGGACCACTGCATCGAGAT
CCTGAGCCACGACAAGGACTACAACAAGGTGAAGCTGTACGAGCACGCCGTGGCCCACA
GCGGCCTGCCCGACAACGCCCGCCGCTAG

mEos3.2
MVSAIKPDMKIKLRMEGNVNGHHFVIDGDGTGKPFEGKQSMDLEVKEGGPLPFAFDILTTAFHY
GNRVFAKYPDNIQDYFKQSFPKGYSWERSLTFEDGGICNARNDITMEGDTFYNKVRFYGTNFP
ANGPVMQKKTLKWEPSTEKMYVRDGVLTGDIEMALLLEGNAHYRCDFRTTYKAKEKGVKLPG
AHFVDHCIEILSHDKDYNKVKLYEHAVAHSGLPDNARR*

This sequence was inserted in-frame into the Clontech (Clontech, Palo Alto, CA) -N1 mammalian expression vector. mEos3.2-N1 contains 3 Cysteine residues, each of which were individually mutated to an Alanine, a Valine, or a Methionine by site directed mutagenesis. Forward primers included:

C101A-5': GAGGACGGCGGCATCGCCAACGCCCGCAACGAC

C101V-5': GAGGACGGCGGCATCGTGAACGCCCGCAACGAC

C101M-5': GAGGACGGCGGCATCATGAACGCCCGCAACGAC

C171A-5': CAACGCCCACTACCGCATGGACTTCCGCACCACC

C171V-5': CAACGCCCACTACCGCGTGGACTTCCGCACCACC

C171M-5': CAACGCCCACTACCGCATGGACTTCCGCACCACC

C195A-5': CCCACTTCGTGGACCACGCCATCGAGATCCTGAG

C195V-5': CCCACTTCGTGGACCACGTGATCGAGATCCTGAG

C195M-5': CCCACTTCGTGGACCACATGATCGAGATCCTGAG

These primers and their complements were then used to make single mutations and then the cysteine-less moxEos3.2.

To create ER-mEos3.2 and ER-moxEos3.2, the coding sequences were PCR amplified and inserted into the *Age*I/*Not*I sites of Clontech N1 vector containing an in-frame bovine prolactin signal sequence 5' to the fluorescent protein coding sequence using the following primers:

F5' GATCACCGGTGGCCACCATGGTGAGCGCCATC

R5' GATCGCGGCCGCCTACAGCTCGTCCTTGCGGCGGGCGTTGTC

Note that the reverse primer appends a KDEL ER retrieval motif at the end of the constructs.

To create CytERM-mEos3.2 and CytERM-moxEos3.2, constructs were cut at *Age*I/*Not*I and the FP fragments were inserted into the *Age*I/*Not*I sites of CytERM-EGFP, replacing the EGFP fluorescent protein coding sequence with the mEos3.2 variants.

Engineering moxDendra2. MoxDendra2 was synthesized by GenScript, replacing Cysteines106 and 176 with Alanines, Cysteine 118 with Threonine (as in mEos3.2) and the consensus N-linked Asparagines with Glutamines. The construct was then inserted in-frame into the Clontech N1 vector.

moxDendra2

MNTPGINLIKEDMRVKVHMEGNVNGHAFVIEGEGKGKPYEGTQTAQLTVKEGAPLPFSYDILTT
AVHYGNRVFTKYPEDIPDYFKQSFPEGYSWERTMTFEDKGIATIRSDISLEGDTFFQNVRFKGT
NFPPNGPVMQKKTLKWEPSTEKLHVRDGLLVGNINMALLLEGGGHYLADFKTTYKAKKVVQLP
DAHFVDHRIEILGQDSDYNKVKLYEHAVARYSPLPSQVW*

ATGAACACCCCGGGAATTAACCTGATCAAGGAGGACATGCGCGTGAAGGTGCACATGGAG
GGCAACGTGAACGGCCACGCCTTCGTGATCGAGGGCGAGGGCAAGGGCAAGCCCTACGA
GGGCACCCAGACCGCCCAGCTGACCGTGAAGGAGGGCGCCCCCCTGCCCTTCAGCTACG
ACATCCTGACCACCGCCGTGCACTACGGCAACCGGGTGTTCACCAAGTACCCCGAGGACA
TCCCCGACTACTTCAAGCAGAGCTTCCCCGAGGGCTACAGCTGGGAGCGCACCATGACCT
TCGAGGACAAGGGCATCGCCACCATCCGCAGCGACATCAGCCTGGAGGGCGACACCTTC
TTCCAGAACGTGCGCTTCAAGGGCACCAACTTCCCCCCCAACGGCCCCGTGATGCAGAAG
AAGACCCTGAAGTGGGAGCCCAGCACCGAGAAGCTGCACGTGCGCGACGGCCTGCTGGT
GGGCAACATCAACATGGCCCTGCTGCTGGAGGGCGGCGGCCACTACCTGGCCGACTTCA
AGACCACCTACAAGGCCAAGAAGGTGGTGCAGCTGCCCGACGCCCACTTCGTGGACCAC
CGCATCGAGATCCTGGGCCAGGACAGCGACTACAACAAGGTGAAGCTGTACGAGCACGC
CGTGGCCCGCTACAGCCCCCTGCCCAGCCAGGTGTGGTAA

To engineer Golgi complex localized GalT-Dendra2 and GalT-moxDendra2, the galactosyltransferase (GalT) signal anchor fragment was cut out of GalT-moxVenus[7] at the *Nhe*I and *Age*I sites, and inserted into the N1 Clontech Dendra2 and moxDendra2 constructs.

To engineer CytERM-moxDendra2, moxDendra2 was subcloned in frame into the *Age*I and *Not*I sites of the CytERM vector.[8]

Cell culture and transfection. U-2 OS and HeLa cells were routinely cultured in RPMI medium (Mediatech, Manassas, VA), supplemented with 5 mM glutamine, penicillin/streptomycin (Invitrogen, Carlsbad, CA), and 10% heat inactivated fetal bovine serum (Hyclone from Thermo Scientific, Rockford, IL) at 37°C in 5% CO_2. For imaging experiments, cells were grown in 8-well LabTek coverglass chambers (Nunc, Rochester, NY). All constructs were transiently transfected for 16-48 h into cells using Lipofectamine 2000 (Invitrogen, of Thermofisher Scientific Waltham, MA) according to the manufacturer's instructions.

Live cell fluorescence imaging. Cells were imaged in phenol red-free RPMI supplemented with 10 mM Hepes (Fisher Scientific, Waltham, MA) and 10% fetal bovine serum. Cells were imaged with an Axiovert 200 widefield fluorescence microscope (Carl Zeiss Microimaging Inc., Thornwood, NY) with a 63X oil NA 1.4 objective, 450-490 excitation/500-550 emission bandpass filter for the green forms of mEos3.2 and Dendra2 and a 565/30 excitation-620/60 emission bandpass filter for the red forms of mEos3.2 and Dendra2. Photoconversion was performed by exciting cells for 30-45 s with a DAPI filter (365 ex and 445/50 emission).

Composite figures were prepared using ImageJ (National Institutes of Health), Photoshop CC 2015 and Illustrator CC 2015 software (Adobe Systems, San Jose, CA).

For imaging photobleaching experiments, cells were grown in eight-well LabTek coverglass chambers (Nunc, Rochester, NY). Cells were imaged in phenol red-free RPMI-1640 freshly supplemented with 10mM Hepes (Thermo Fisher Scientific) and 10% fetal bovine serum. Live cells were imaged on a 37ºC environmentally controlled chamber of a confocal microscope system (Zeiss LSM-5 LIVE microscope with Duoscan attachment; Carl Zeiss MicroImaging, Inc.) with a 63x1.4 NA oil objective and a 489 nm 100mW diode laser with a 495–555 and 520–555nm bandpass filter for GFP or 561nm diode laser with a 565 nm longpass filter for mCherry. Image analysis and composite figures were prepared using ImageJ (National Institutes of Health; Bethesda, MD), and Prism (GraphPad Software, LaJolla, CA).

Immunoblots. Total cell lysates for immunoblotting were prepared in 1% SDS, 0.1 M Tris, pH 8.0 using HeLa cells in 12 well plates at 80-90% confluence. Proteins were separated using 12% Tris-tricine gels, transferred to nitrocellulose, probed with anti-mEos2 antibody (A010-mEOS2) (Badrilla from Leeds Innovation Centre, Leeds UK)) followed by a secondary antibody (horseradish peroxidase-labeled anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA))., and developed using enhanced chemiluminescent reagents (Pierce, Rockford, IL), and exposed to X-ray film.

Table 1. Photochemical properties of Dendra2 and moxDendra2

	Dendra2		moxDendra2	
	before activation	after activation	before activation	after activation
Excitation maximum, nm	490	551	490	551
Emission maximum, nm	504	571	504	571
Quantum yield	0.50	0.55	0.50	0.55
Extinction coefficient, M^{-1} cm^{-1}	39,700	26,600	50,300	31,200
Half-time of photostability, s	30	1,050	10	330
Half-time of photoswitching, s	60		45	
Contrast of photoswitching, fold	greater than 1,000		greater than 1,000	
Molecular brightness*	0.60	0.44	0.74	0.50

* Brightness relative to EGFP (extinction coefficient = 56,000 M^{-1} cm^{-1} and quantum yield = 0.6).

Supplementary Figures

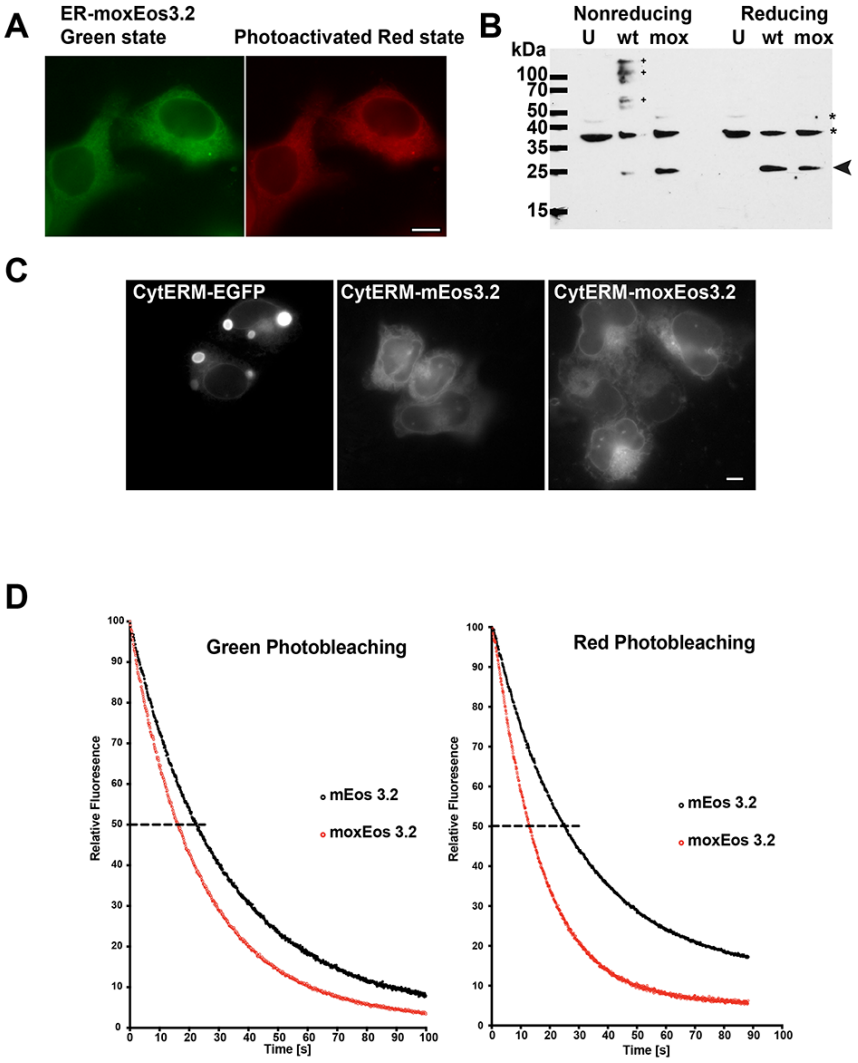

Fig. S1. mEos3.2 and moxEos3.2 characterization in cells. (A) HeLa cells expressing ER-moxDendra2 and imaged with a widefield microscope. Cells exhibit a characteristic endoplasmic reticulum pattern of a tubular network and nuclear envelope. Cells were photoconverted with excitation with a DAPI filter and then imaged in the red channel, that exhibited robust photoactivation. Scale bar = 10 μm. (B) HeLa cells transiently transfected with no plasmid (U), ER-mEos3.2 (wt) or ER-moxEos3.2 (mox) were lysed and separated by SDS-PAGE by reducing or nonreducing conditions. Nonspecific bands are indicated by *. Arrowhead indicates expected band position at 25 kDa for a monomer in the ER, oligomerized mEos3.2 species are indicated by +. (C) EGFP (positive control), mEos3.2 and moxEos3.2 were fused to the CytERM reporter and imaged by fluorescence microscopy to detect whorls and other evidence of OSER formation, and thus, nonmonomeric protein behavior. While EGFP CytERM readily forms whorls, no whorls are observed for mEos3.2 or moxEos3.2. Scale bar = 5 μm. (D) HeLa cells transiently transfected with ER-mEos3.2 or ER-moxEos3.2 were imaged with a laser scanning confocal microscope continuously with either a 488 nm laser (green channel) or 561 nm laser (red channel). Loss of mean fluorescence intensity of cells was quantitated and plotted for at least 8 cells for each condition.

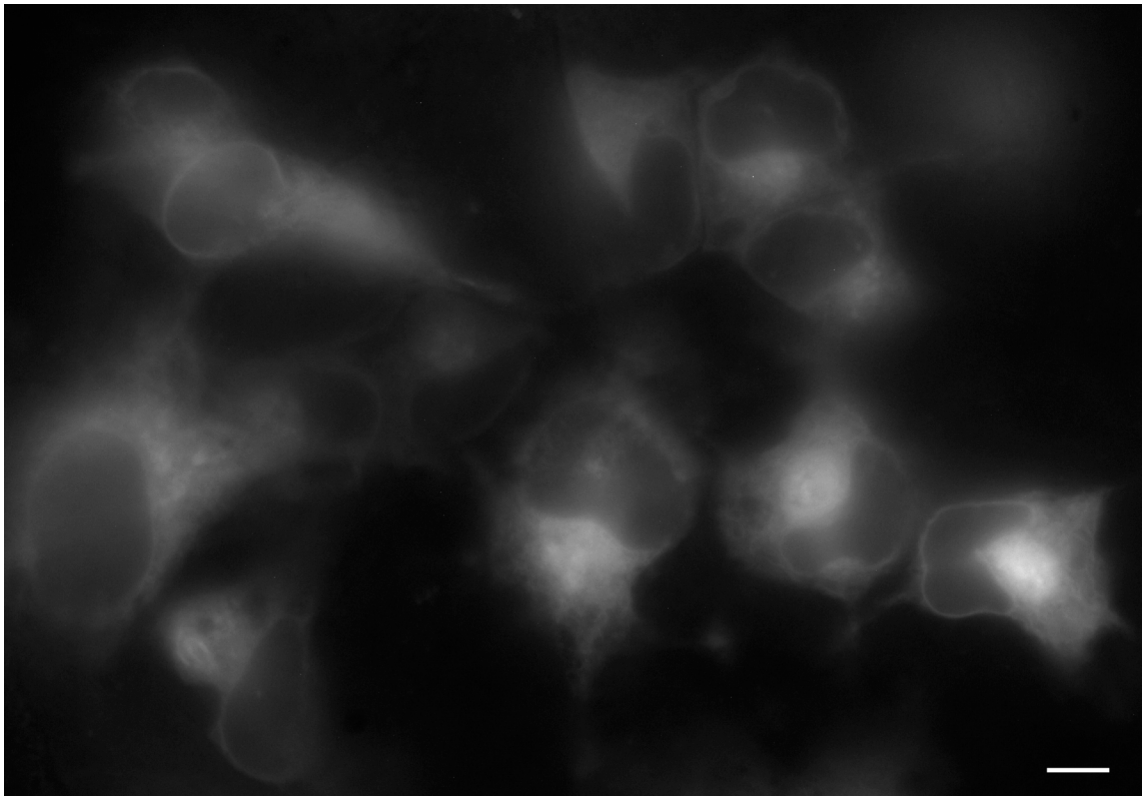

Fig. S2. CytERM-moxDendra2 reveals the modified protein retains its monomeric properties. The construct was expressed in HeLa cells for 24 h. The CytERM-moxDendra2 construct localizes to the Endoplasmic Reticulum (ER) and nuclear envelope. No membrane accumulations on the nuclear envelope (karmellae) or within the ER (whorls) were observed in the entire field of cells. The absence of these structures indicates moxDendra2 does not appear to oligomerize Therefore, as with the parent Dendra2, moxDendra2 appears to be robustly monomeric. Scale bars = 10 μm.

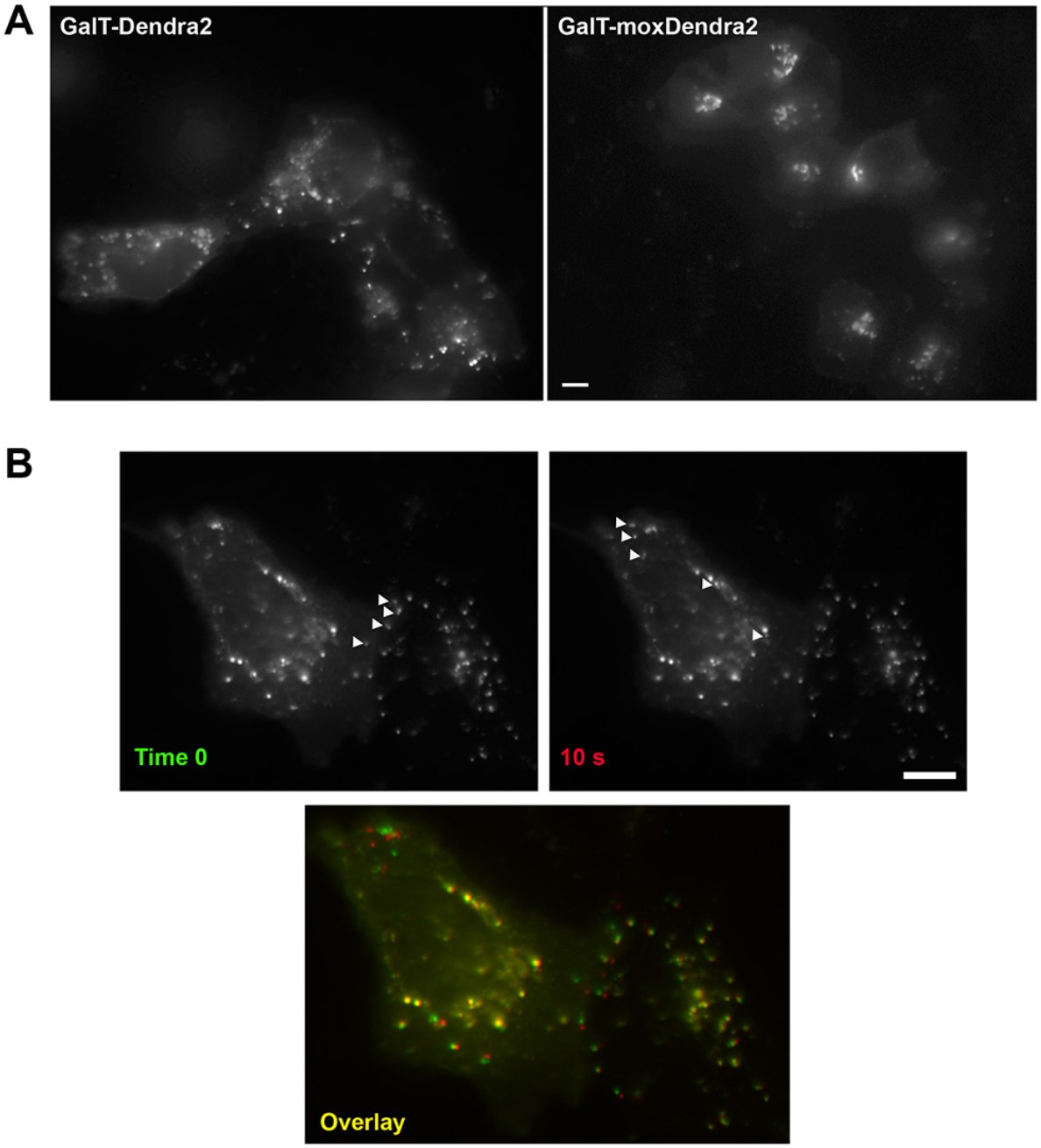

Fig. S3. Localization of Golgi complex marker GalT-fusions to Dendra2 and moxDendra2 in U-2 OS cells. (A) Note that the Dendra2 constructs are observed in vesicular structures throughout the cell, while the moxDendra2 construct is robustly localized to compact perinuclear stacked membrane structures characteristic of the Golgi complex. Scale bar = 5 μm. (B) The vesicles for the Dendra2 were observed to move throughout the cell. The same cell was imaged at time 0 and 10 s later. The two images were superimposed with time 0 in a green channel and 10 s in a red channel. Arrowheads indicate vesicles that clearly move and differ in positions between the two images. Scale bar = 10 μm.

Supplementary References

1. D. M. Chudakov, V. V. Verkhusha, D. B. Staroverov, E. A. Souslova, S. Lukyanov and K. A. Lukyanov, *Nat Biotechnol*, 2004, **22**, 1435-1439.
2. D. M. Chudakov, S. Lukyanov and K. A. Lukyanov, *Biotechniques*, 2007, **42**, 553, 555, 557 passim.
3. A. R. Williams, S. A. Winfield and J. N. Miller, *Analyst*, 1983, **108**, 1067.
4. H. C. Lee and H. D. Bernstein, *Proc Natl Acad Sci U S A*, 2001, **98**, 3471-3476.
5. D. E. Aronson, L. M. Costantini and E. L. Snapp, *Traffic*, 2011, **12**, 543-548.
6. J. Haas, E. C. Park and B. Seed, *Curr Biol*, 1996, **6**, 315-324.
7. L. M. Costantini, M. Baloban, M. L. Markwardt, M. Rizzo, F. Guo, V. V. Verkhusha and E. L. Snapp, *Nat Commun*, 2015, **6**, 7670.
8. L. M. Costantini, M. Fossati, M. Francolini and E. L. Snapp, *Traffic*, 2012, **13**, 643-649.