

Expanded View Figures

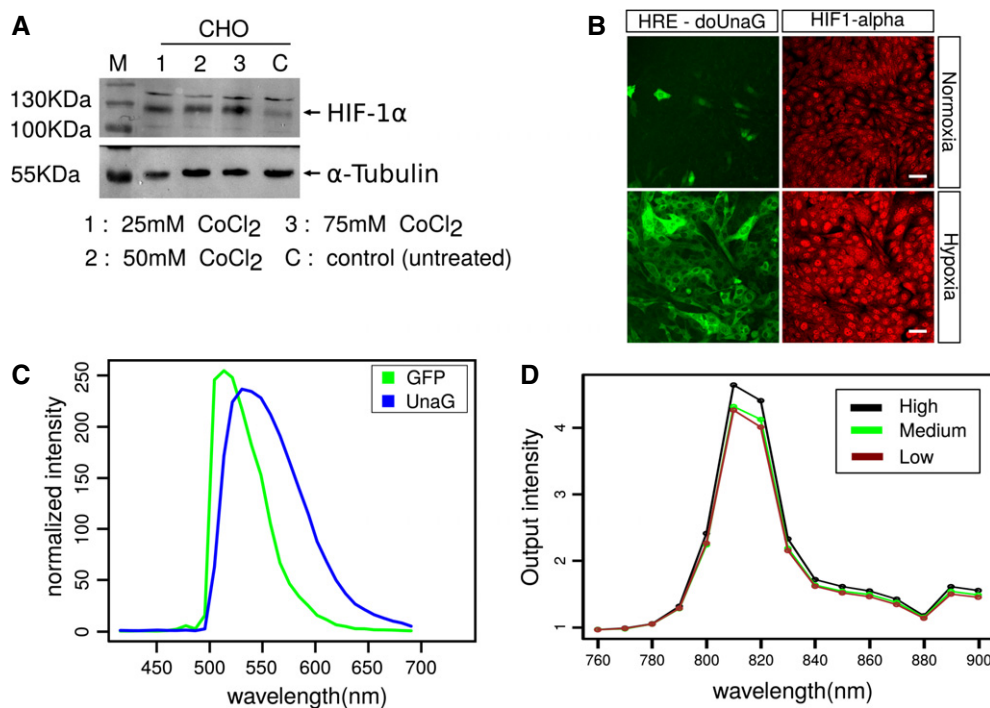


Figure EV1. Induction of HIF-1 α in CHO cells and spectral properties of UnaG.

- A** Culture in the presence of the hypoxia-mimetic CoCl₂ resulted in an efficient induction of HIF-1 α stabilization in CHO cells. CHO cells were plated in 5-cm round bottom dishes at 60% confluency, and CoCl₂ was added to the dishes at the indicated concentrations. 24 h after the treatment, cells were harvested and lysed and HIF-1 α expression was analyzed by Western blotting. M, molecular mass marker.
- B** Stabilization of HIF-1 α correlated with the induction of doUnaG fluorescence in cells cultured for 24 h in the presence of 1% oxygen. HIF-1 α was visualized by immunostaining. Scale bar, 50 μ m.
- C, D** Characterization of the spectral properties of dUnaG expressed in CHO cells. (C) Comparison of the fluorescence properties of EGFP and UnaG expressed in CHO cells in single photon microscopy. CHO cell bulk cultures expressing either dUnaG or dEGFP were plated in IBIDI chambers and incubated for 24 h under hypoxia (1% oxygen) before imaging. Cultures expressing either fluorescent protein were then excited at 488-nm wavelength and emission fluorescence was recorded using the lambda scan mode on a Zeiss LSM780 confocal microscope. Plotted is the normalized fluorescence output for dEGFP and dUnaG, showing the wider fluorescence spectrum of dUnaG as compared to dEGFP. (D) dUnaG is efficiently excited at 810 nm in multiphoton microscopy. Excitation wavelength scan of dUnaG-expressing CHO cells previously kept under hypoxia for 24 h using a multiphoton microscope. The wavelength of excitation light produced by a titanium sapphire laser was increased from 760 to 900 nm, and a single image was acquired at 10-nm step width using the wavelength scan mode of the LaVision Biotech Inspector Pro software. Relative fluorescence values were normalized to the square of the laser output. Three cells with high, low, and medium expression of UnaG were selected for analysis.

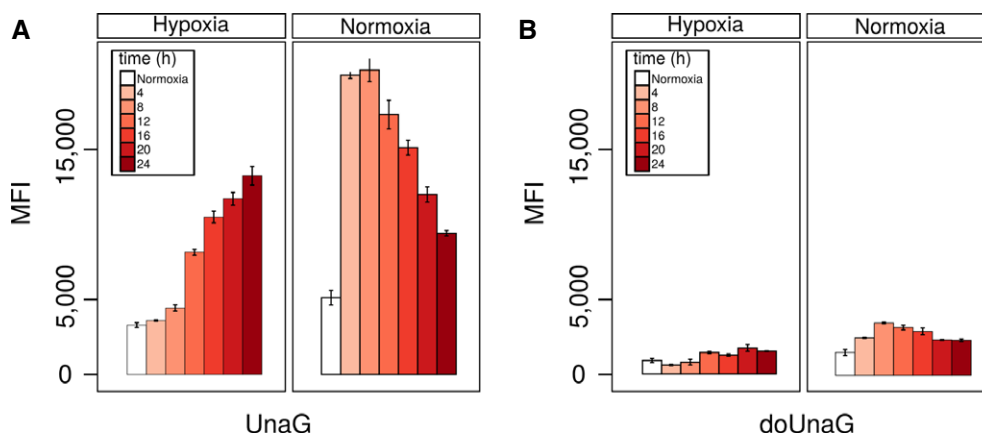


Figure EV2. Cytometric analysis of the expression kinetics of the two UnaG-based hypoxia sensor variants HRE-UnaG and HRE-doUnaG.

A, B Flow cytometric analysis of the induction and decay kinetics of the fluorescence of UnaG (A) and doUnaG (B) in CHO cells stably expressing either variant. Open bars represent control cultures grown under normoxia. Solid bars indicate cultures grown for 4, 8, 12, 16, 20, or 24 h under hypoxia (1% oxygen) to activate the sensor or for the same time under normoxia (21% oxygen) to deactivate the sensor. Deactivation was initiated after the cells were grown under hypoxia (1% oxygen) for 24 h. Error bars represent SE.

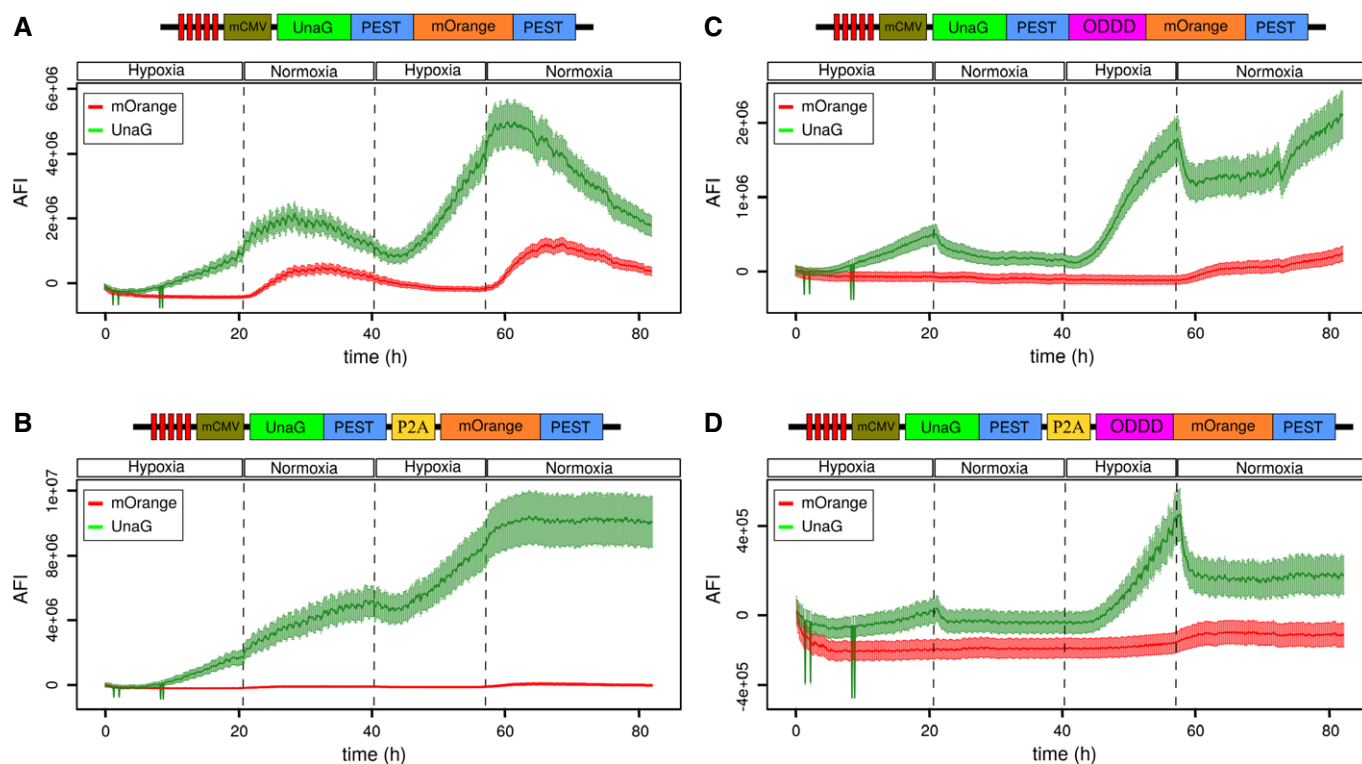
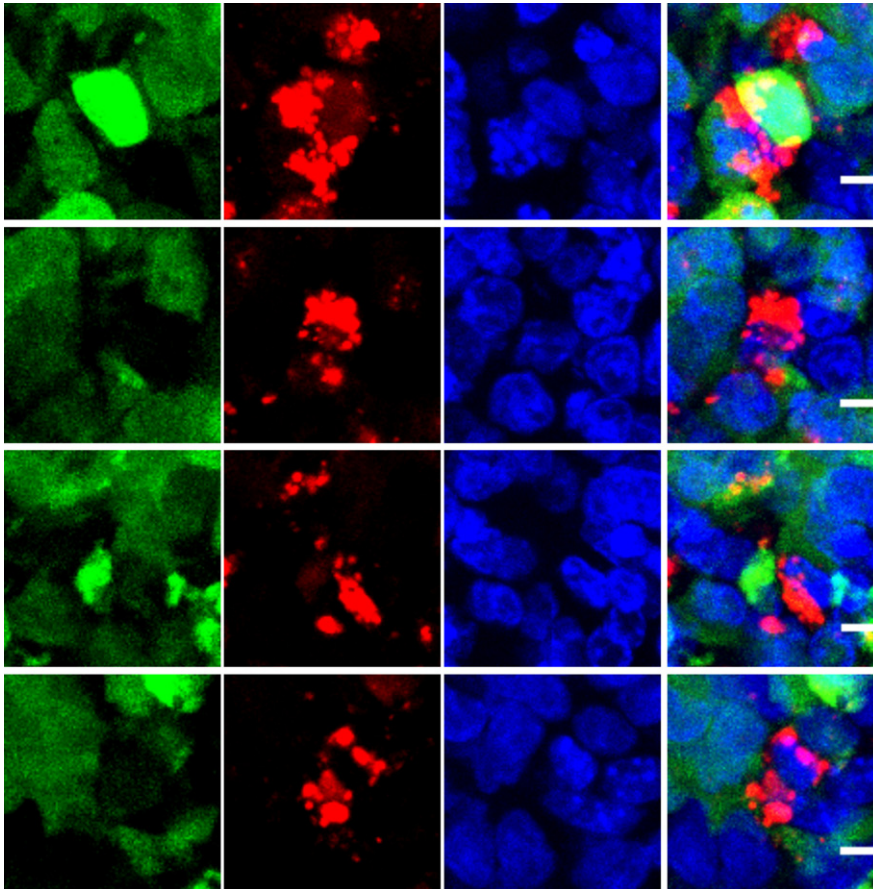


Figure EV3. Characterization of several variants of a destabilized UnaG-mOrange hypoxia-reoxygenation combination sensor.

A schematic representation of the different sensor constructs is shown on top of each panel. In all constructs, expression is driven by the 5x HRE-mCMV promoter. In-frame fusion to a PEST sequence from mouse ornithine carboxylase or the oxygen-dependent degradation domain sequence (ODDD) from HIF-1 α (aa 338-608) was used to destabilize the proteins. The panels underneath depict the microscopic assessment of the normalized, averaged fluorescence intensity (AFI) in CHO cells stably transfected with the dUnOHR sensor variants. Hypoxia was induced by incubation in 1% oxygen for 20 h, and then, cultures were switched to normoxia for 20 h, followed by another 17-h hypoxia and finally 24 h of normoxia again. For optimal comparison, live cell imaging of all variants depicted was performed in parallel in various wells of a single IBIDI 8-well chamber slide. Plotted is the average of the mean \pm SEM.

- A The UnaG-PEST-mOrange-PEST fusion in-frame protein is induced under hypoxia, while mOrange fluorescence becomes detectable after switch to normoxia. This construct behaved similar to the dUnOHR (UnaG-mOrange-PEST) sensor. The increase in both green and orange fluorescence was limited under normoxia by the subsiding promoter activity. This behavior was repeated in subsequent hypoxia-normoxia cycles.
- B The UnaG-PEST-P2A-mOrange-PEST sensor was induced under hypoxia, but failed to decay under normoxia; simultaneously, orange fluorescence failed to become detectable. UnaG fluorescence further increased during the subsequent hypoxia cycle and then plateaued, while orange remained undetectable; hence, this sensor was not followed further.
- C, D UnaG was induced in both UnaG-PEST-ODDD-mOrange-PEST and UnaG-PEST-P2A-ODDD-mOrange-PEST, was reduced under normoxia, and fluorescence kinetics were similar to the UnaG-PEST-ODDD (doUnaG) fusion protein (Fig 2B). In both sensors, orange fluorescence remained largely undetectable, severely limiting their applicability.

**Figure EV4. Increased cell fragmentation of reoxygenated cells.**

High-resolution imaging of a tumor formed by intracranial injection of 500 Gli36 cells stably transfected with the dUnOHR sensor. Tissue was counterstained by Hoechst 33342. Intense mOrange-containing aggregates were observed preferentially in the cells that displayed signs of nuclear and cytoplasmic fragmentation indicative of apoptotic decay and located at the edge of hypoxic areas. Scale bar, 5 μ m.