Supplementary figures

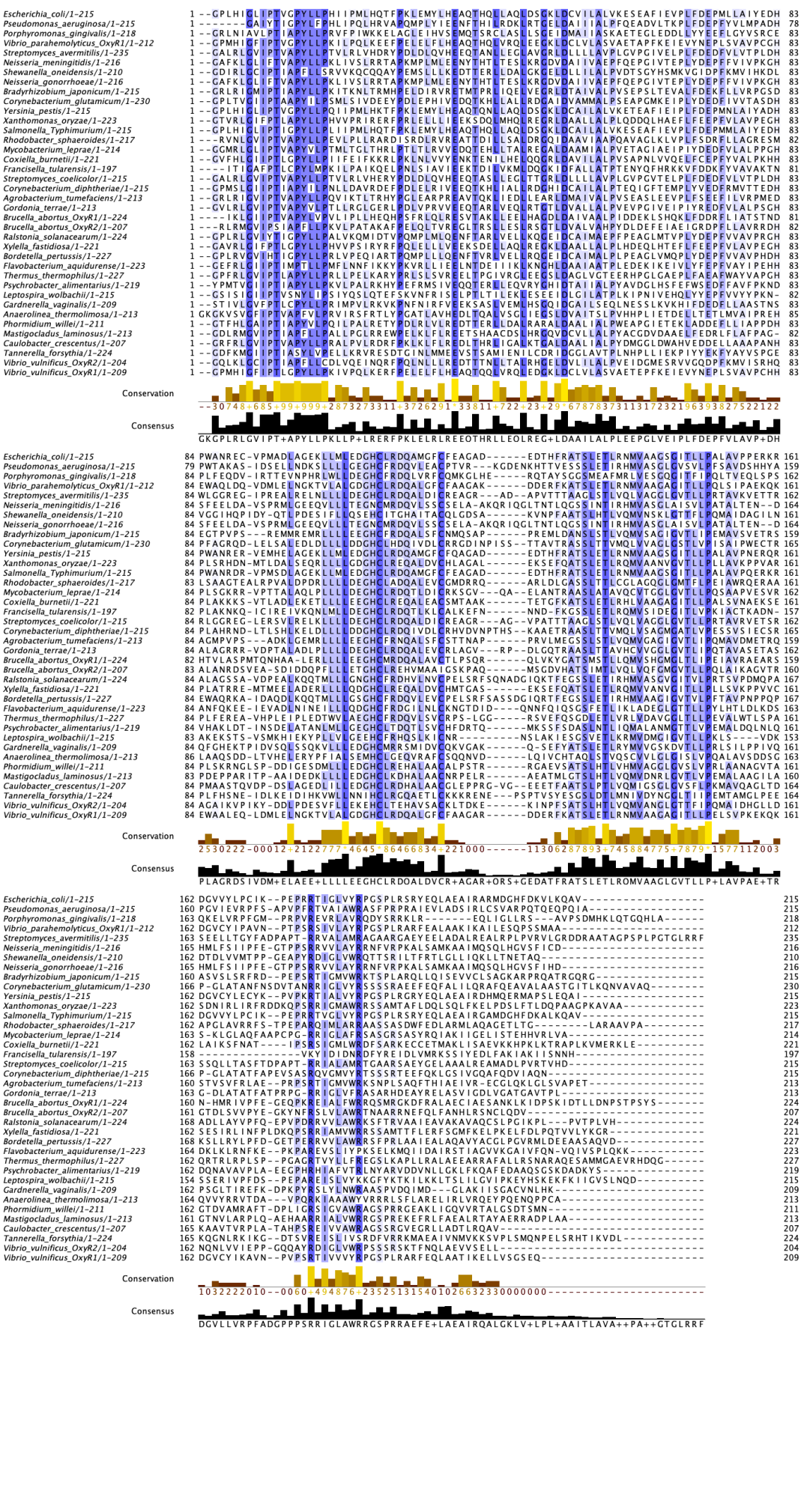

Figure S1. Multiple sequence alignment of 38 OxyR regulatory domain sequences, related to Figure 1. The 38 OxyR regulatory domain sequences were taken from Uniprot or Uniparc database (http://www.uniprot.org), with some sequences taken from Genbank (highlighted next to the accession number): *Escherichia coli*, P0ACQ4; *Pseudomonas aeruginosa*, Q9HTL4; ***Porphyromonas gingivalis,* Q7MXD3**; *Vibrio parahemolyticus* OxyR1, Q87L60; ***Streptomyces avermitilis*, Q82IC7**; ***Neisseria meningitidis*, A1KRK4**; *Shewanella oneidensis*, Q8EHA1; *Neisseria gonorrhoeae*, B4RR00; *Bradyrhizobium japonicum*, A0A023XTM7; ***Corynebacterium glutamicum*, Q8NP91**; *Yersinia pestis*, Q7CL10; *Xanthomonas oryzae*, Q5GWM0; *Salmonella enterica* bv. Typhimurium, Q7CPB9; *Rhodobacter spheroides*, Q3J2P7; *Mycobacterium leprae*, P52678; *Coxiella burnetii*, Q83BM7; ***Francisella tularensis*, Q5NHB0**; *Streptomyces coelicolor*, Q9RN71; *Corynebacterium diphtheriae*, H2GUE0; *Agrobacterium tumefaciens*, A9CGX9; *Gordonia terrae*, H5UA91; ***Brucella abortus* OxyR1, F8WJR3**; *Brucella abortus* OxyR2, A0A0F6AUI6; ***Ralstonia solanacearum*, F6FYN0**; *Xylella fastidiosa*, Q87DD8; *Bordetella pertussis*, Q7VXW3; *Flavobacterium aquidurense*, A0A0Q0XTK2; *Thermus thermophilus*, Q5SM20; *Psychrobacter alimentarius*, A0A144Q2V7; *Leptospira wolbachii*, R8ZZY6; *Gardnerella vaginalis*, A0A1H1L1G5; ***Anaerolinea thermolimosa*, GAP06981.1 (Genbank)**; *Phormidium willei*, A0A168SMG3; ***Mastigocladus laminosus*, WP_072046693 (Genbank)**; *Caulobacter crescentus*, Q9A269; *Tannerella forsythia*, G8UJV8; ***Vibrio vulnificus* OxyR2, A0A087I947**; *Vibrio vulnificus* OxyR1, A0A1V8MGK0; Names highlighted in bold indicate the 11 OxyR regulatory domain sequences tested, with *Neisseria meningitidis* (in red) finally chosen for HyPer7. Multiple sequence alignment was performed with ClustalW; included in the MEGA software package (Kumar et al., 2016). Color intensity represents sequence identity, and the conserved residues that belong to the OxyR catalytic pentad are highlighted in red. The figure was created using Jalview (Waterhouse et al., 2009).

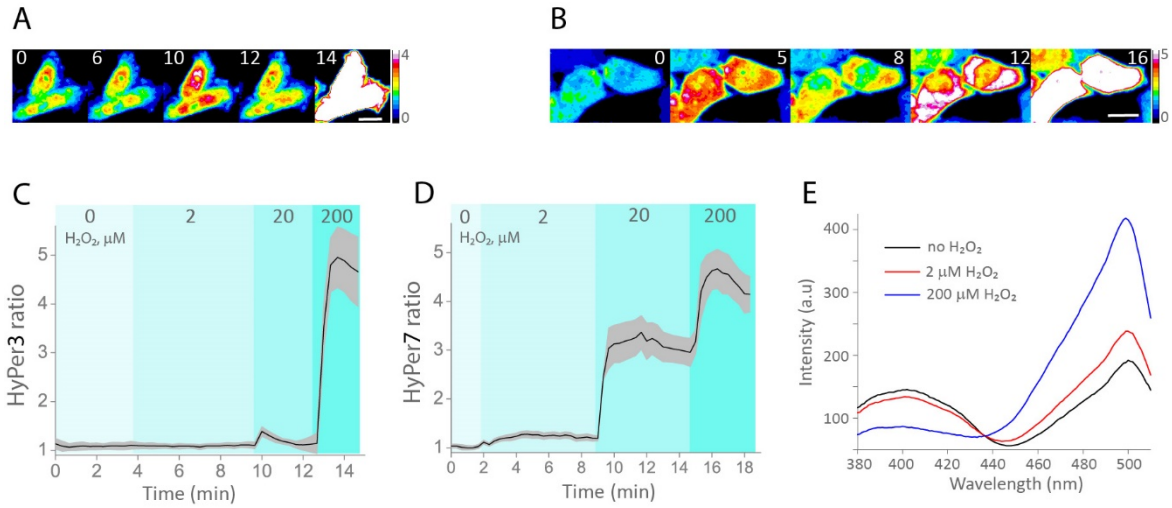

Figure S2. Sensitivity of HyPer7 to exogenous H_2O_2 in cells, related to Figure 1. HyPer3 or HyPer7 were transiently expressed in HeLa-Kyoto cells (A-D). Ratiometric images of the cells expressing HyPer3 (A) or HyPer7 (B) exposed to increasing concentrations of H_2O_2. Numbers indicate time in minutes following H_2O_2 addition. Scale bars are 15 µm. Time course of HyPer3 (C) and HyPer7 (D) ratio changes upon the sequential addition of 2, 20 or 200 mM H_2O_2. (E) Similarly, HyPer7 reacts to 2 µM exogenous H_2O_2 in the *E. coli* cytoplasm. Excitation spectrum of HyPer7 recorded at 520 nm emission.

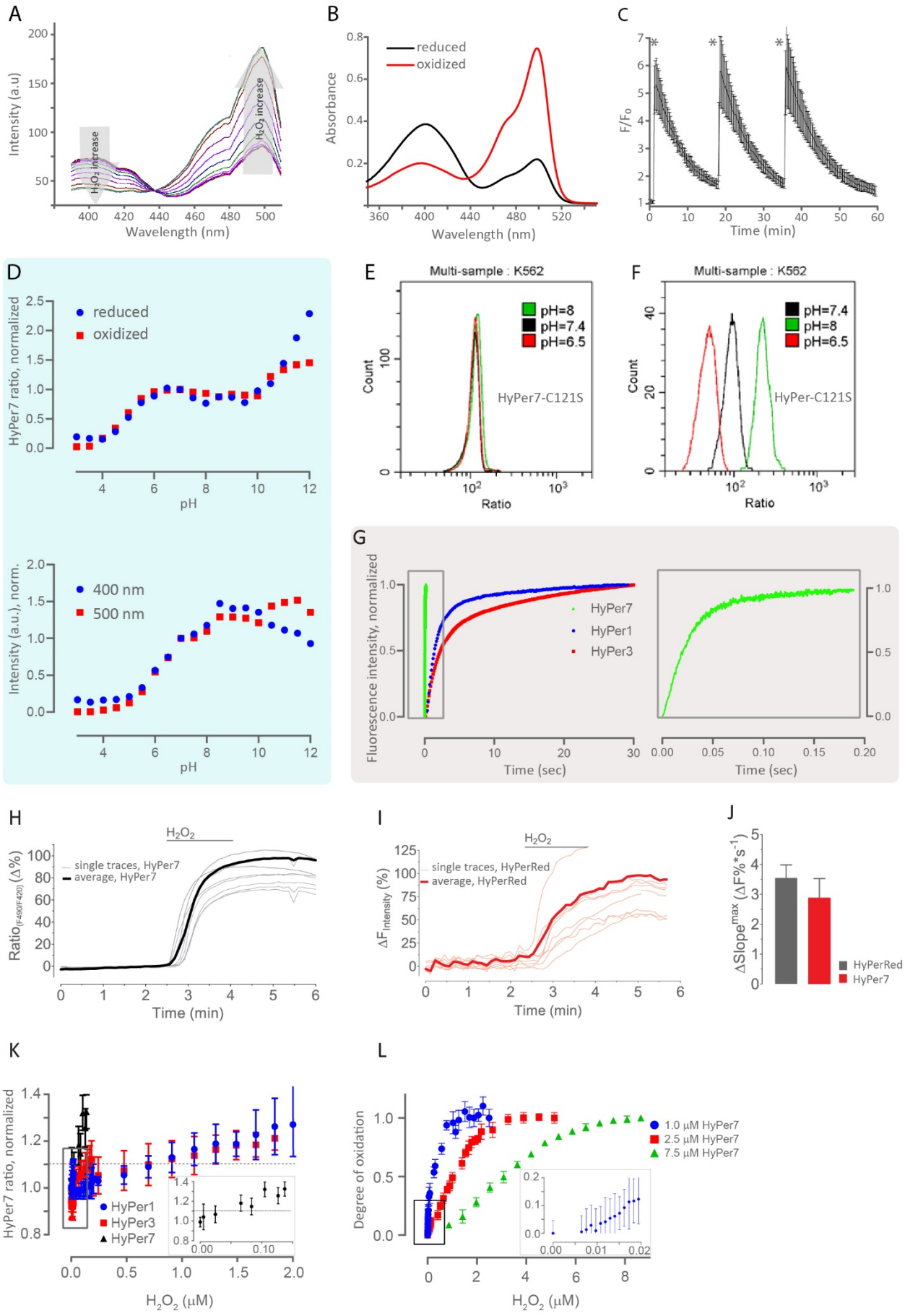

Figure S3. *In vitro* characterization of HyPer7, related to Figure 2. (A) Excitation spectrum of HyPer7 upon gradual increase of H_2O_2 concentration. (B) Absorption spectrum of HyPer7. (C) HyPer7 in the cytosol of HeLa-Kyoto cells upon three consecutive additions of an excess of H_2O_2. Each star marks an

addition of 100 μM H_2O_2. Notice complete reduction of the probe. (D) Upper graph: full pH range titration of the HyPer7 ratio (excitation maxima at 500 nm/400 nm), normalized to the value at pH 7.0 of reduced (in the presence of 5 mM TCEP) and oxidized (in the presence of 100 μM H_2O_2). Lower graph: pH dependencies of the intensities of the 400 nm and 500 nm excitation maxima of oxidized HyPer7, normalized to the values of the intensity at pH 7.0. (E) Due to its high pH stability HyPer ratio does not change upon exposure of cells to media with different pH. Flow cytometry histograms display HyPer7 ratio signals in K562 cells. This is in sharp contrast to HyPer (F) that demonstrates large signal shifts upon media acidification or alkalization. To prevent possible signal changes due to probe oxidation the C121S versions of the probes were used. (G) Representative progress curves of the reaction of HyPer 1, 3 and 7 (left graph) as recorded with a stopped-flow spectrophotometer. The progress curve for HyPer7 (marked with a rectangle on the left graph) is portrayed on the right graph with a different time scale. In each case, 2.5 μM protein pre-reduced with 62.5 μM TCEP overnight in 100 mM sodium phosphate buffer, pH 7.4 with 0.1 mM DTPA were used. The final concentration of H_2O_2 in each case was 250 μM. The curves represent the increase of the fluorescence intensity at λ_{ex} = 495 nm, with emission detection at λ_{em} = 515 nm. The intensity values were normalized to the maximum intensity reached. (H-J) Kinetic differences between the HyPer7 and HyPerRed in Eahy.926 cells co-expressing both probes. (H) Average curve (bold black) represents HyPer7 ratio in response to 50 μM H_2O_2. Light grey curves represent individual cell responses. (I) Average curve (bold red) of HyPerRed in response to 50 μM H_2O_2. Light red curves represent individual cell responses. (J) Bars represent maximum slope (on kinetics) of HyPer7 (black bar, n=3/11) and HyPerRed (red bar, n=3/11) in response to 50 μM H_2O_2. Mean values are shown ±SEM, $*p < 0.05$ versus control using unpaired *t*-test. (K) The response of HyPer 1, 3 and 7 (2.5 μM each) to increasing amounts of H_2O_2. The y-axis represents the ratio between the 500 and 400 nm excitation maxima, normalized to the ratio prior to the addition of oxidant. Each point represents the mean of three repeats; the error bars denote the standard deviation. The inset depicts the lower nM range for HyPer7. The horizontal dotted line on each graph corresponds to an increase of the ratio by 0.1 a.u., which was used to compare the sensitivities between the different HyPer versions. (L) The response of HyPer7 of the indicated concentrations to increasing concentrations of H_2O_2. The y-axis represents the degree of oxidation calculated taking the 500/400 ratio at the plateau as 100% oxidation and the ratio before H_2O_2 addition as 0% oxidation. Each point represents the mean of three repeats; the error bars denote the standard deviation. The inset depicts the lower nM range for HyPer7 at a concentration of 1 μM.

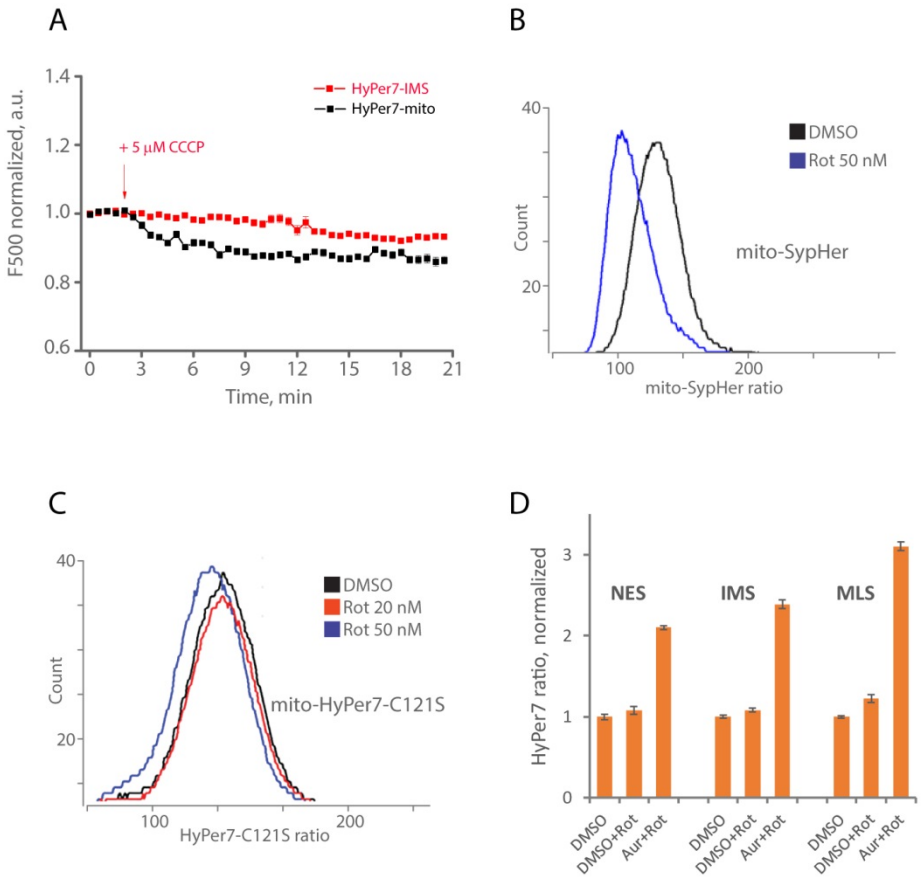

Figure S4. Mitochondrial H_2O_2 production, related to Figure 3. (A) Addition of the uncoupler CCCP induces a decrease in F500 fluorescence of HyPer7 located to the matrix, but not of the HyPer7-IMS indicating proper localization of both probes. (B) pH-sensitivity of HyPer leads to a decrease of mito-HyPer ratio upon rotenone addition due to acidification of the mitochondrial matrix. This signal would mask the HyPer signal change due to H_2O_2 production in the matrix. (C) In contrast to HyPer, the pH-stable HyPer7 signal does not change upon acidification of the matrix. To prevent possible signal changes due to the probe oxidation C121S versions of the probes were used. (D). The lack of HyPer7 ratio change in the cytosol (NES, nucleus exclusion signal), and the mitochondrial intermembrane space (IMS), but its increase in the mitochondrial matrix (MLS, mitochondrial localization signal = mito) upon rotenone (50 nM) addition indicates that H_2O_2 is released by Complex I at the matrix side. Auranofin potentiates H_2O_2 release induced by rotenone, implying a role of the thioredoxin system in preventing H_2O_2 spread from the matrix. The data are the results of flow cytometry analysis. Each bar represents the mean of three replicates and the error bars denote the standard deviation.

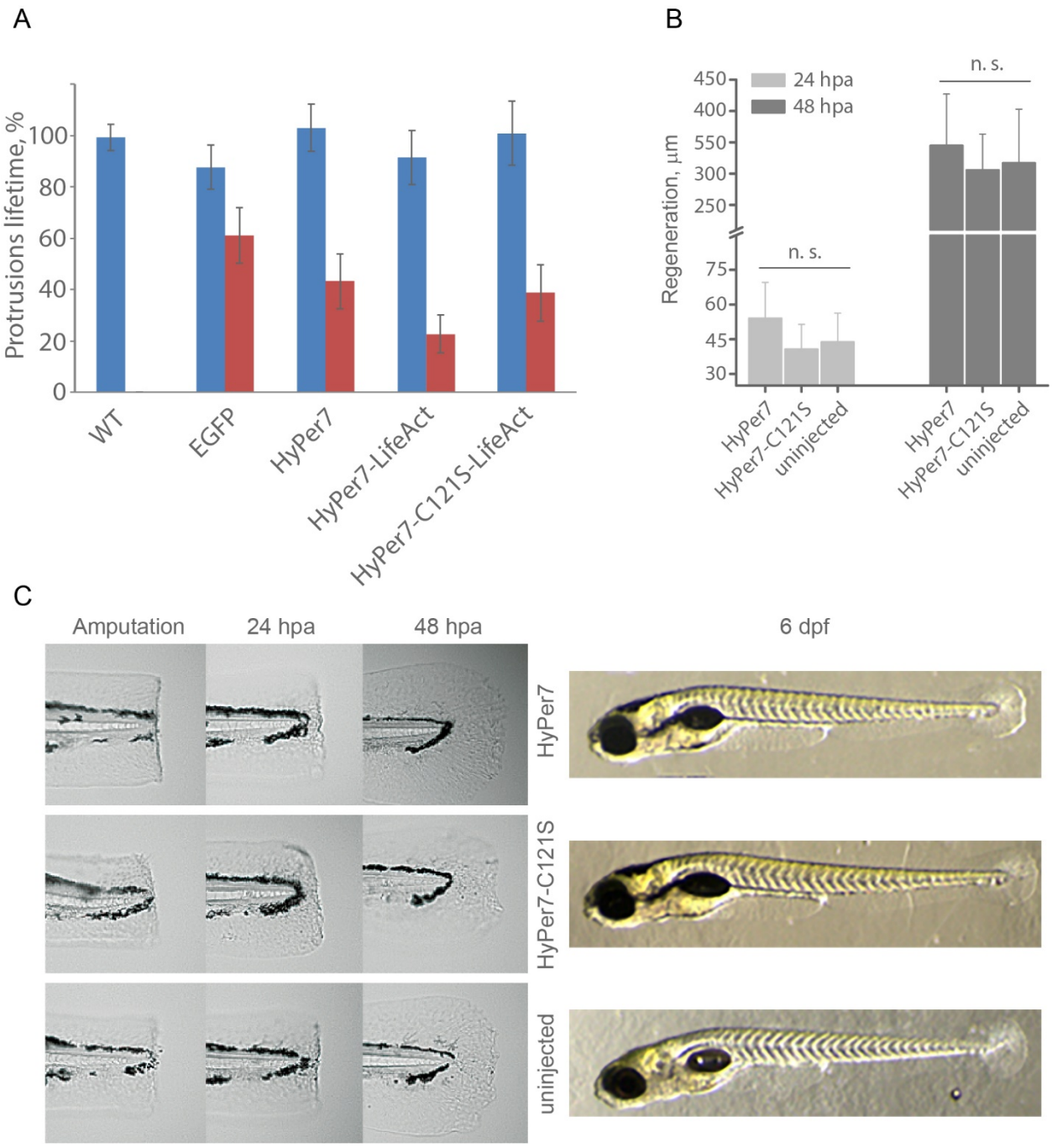

Figure S5. HyPer7 does not affect cell motility and regeneration, related to Figures 4,5. (A) Comparison of protrusion lifetime between WT NIH-3T3 cells and cells expressing various versions of HyPer7 or EGFP. Blue bars – cells with low-to-moderate expression levels. Red bars – cells with high expression levels (top 15% brightest cells). (B, C) Impact of HyPer7 or HyPer7-C121S expression on regeneration in zebrafish larvae. Zebrafish embryos were injected with 100 ng/µL HyPer7 or SypHer7 mRNAs at the one-cell stage and a tail fin amputation assay was performed on 2 dpf larvae. Regeneration was then quantified by measuring the length of the tail fin after the notochord. (B) Regeneration quantifications (values ± SD; n ≥ 20 embryos/condition; ns, non significant) (C) Images of larvae tail fin amputated at 24 and 48 hpa, and images of 6 dpf animals.