

## APPENDIX

### **Thioredoxin reductase controls the capacity of peroxiredoxins to limit mitochondrial H<sub>2</sub>O<sub>2</sub> release**

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## APPENDIX TABLES

**Appendix Table S1. Antibodies**

| Antibody | Source         | Identifier  |
|----------|----------------|-------------|
| roGFP2   | Self -made     | -           |
| FLAG     | Sigma-Aldrich  | F3165       |
| HIF1a    | BD Bioscience  | 610959      |
| Hsp70    | Santa Cruz     | sc-66048    |
| PRDX1    | Sigma-Aldrich  | HPA007730   |
| PRDX2    | Sigma-Aldrich  | WH0007001M1 |
| PRDX3    | Sigma-Aldrich  | HPA041488   |
| PRDX5    | Sigma-Aldrich  | HPA037915   |
| PRDX6    | Sigma-Aldrich  | HPA006983   |
| TXN      | Cell signaling | 24295       |
| TXNRD1   | Cell signaling | 151405      |

**Appendix Table S2. Plasmids**

| Plasmid         | Backbone      | Insert                                                                      | Source      |
|-----------------|---------------|-----------------------------------------------------------------------------|-------------|
| Cyto-HyPer3     | pC1           | HyPer3                                                                      | Addgene     |
| Matrix-HyPer3   | pcDNA5/FRT/TO | Su9 <sup>MTS(1-69)</sup> -HyPer3                                            | This study  |
| SypHer          | pcDNA5/FRT/TO | SypHer                                                                      | This study  |
| Mito-SypHer     | pC1           | 2x Cox8 <sup>MTS(1-65)</sup> -SypHer                                        | Addgene     |
| Cyto-HyPer7     | pCS2+         | HyPer7-RevNES(3x)                                                           | V. Belousov |
| Matrix-HyPer7   | pCS2+         | 2xCox8 <sup>MTS(1-25)</sup> -HyPer7                                         | V. Belousov |
| IMS-HyPer7      | pCS2+         | Smac <sup>MTS(1-78)</sup> -HyPer7                                           | V. Belousov |
| OMM-HyPer7      | pCS2+         | Tom20 <sup>MTS(1-29)</sup> -linker<br>(LEA(EAAAK) <sub>4</sub> ALE )-HyPer7 | This study  |
| PRDX2-HA        | pcDNA5/FRT/TO | PRDX2-HA                                                                    | This study  |
| Su9-DAO         | pcDNA5/FRT/TO | Su9 <sup>MTS(1-69)</sup> -RyDAO-FLAG                                        | This study  |
| Su9-DAO         | Piggy bac     | Su9 <sup>MTS(1-69)</sup> -RyDAO-FLAG                                        | This study  |
| roGFP-Prx1      | pcDNA5/FRT/TO | roGFP2-scPrx1                                                               | This study  |
| Mito-roGFP-Prx1 | pcDNA5/FRT/TO | Su9 <sup>MTS(1-69)</sup> -roGFP2-scPrx1                                     | This study  |

**Appendix Table S3.** Cell lines

| Cell line                                                     | Plasmid            | Construct                                   | Tag          | source              |
|---------------------------------------------------------------|--------------------|---------------------------------------------|--------------|---------------------|
| HeLa                                                          | -                  | -                                           | -            | -                   |
| HeLa matrix-DAO                                               | PiggyBac           | Su9 <sup>MTS(1-69)</sup> -RgDAO             | -FLAG        | This study          |
| Flp-In T-Rex-293 MOCK                                         | pcDNA5             | Empty                                       | -            | Fischer et al. 2013 |
| Flp-In T-Rex-293 Grx1-roGFP2                                  | pcDNA5             | hsGrx1-roGFP2                               | -            | Fischer et al. 2013 |
| Flp-In T-Rex-293 matrix-Grx1-roGFP2                           | pcDNA5             | Su9 <sup>MTS(1-69)</sup> - hsGrx1-roGFP2    | -            | Fischer et al. 2013 |
| Flp-In T-Rex-293 matrix-DAO                                   | pcDNA5             | Su9 <sup>MTS(1-69)</sup> -RgDAO             | -FLAG        | This study          |
| Flp-In T-Rex-293 PRDX1 CRISPR KO                              | -                  | -                                           | -            | This study          |
| Flp-In T-Rex-293 PRDX2 CRISPR KO                              | -                  | -                                           | -            | This study          |
| Flp-In T-Rex-293 PRDX6 CRISPR KO                              | -                  | -                                           | -            | This study          |
| Flp-In T-Rex-293 PRDX6 KO + Su9-DAO                           | pcDNA5             | Su9 <sup>MTS(1-69)</sup> -RgDAO             | -FLAG        | This study          |
| Flp-In T-Rex-293 PRDX1 + PRDX2 CRISPR KO                      | -                  | -                                           | -            | This study          |
| Flp-In T-Rex-293 PRDX1 CRISPR KO + Su9-DAO                    | PiggyBac           | Su9 <sup>MTS(1-69)</sup> -RgDAO             | -FLAG        | This study          |
| Flp-In T-Rex-293 PRDX2 CRISPR KO + Su9-DAO                    | PiggyBac           | Su9 <sup>MTS(1-69)</sup> -RgDAO             | -FLAG        | This study          |
| Flp-In T-Rex-293 PRDX1 + PRDX2 CRISPR KO + Su9-DAO            | PiggyBac           | Su9 <sup>MTS(1-69)</sup> -RgDAO             | -FLAG        | This study          |
| Flp-In T-Rex-293 PRDX1 + PRDX2 CRISPR KO + PRDX2-HA + Su9-DAO | pcDNA5<br>PiggyBac | PRDX2-HA<br>Su9 <sup>MTS(1-69)</sup> -RgDAO | -HA<br>-FLAG | This study          |

**Appendix Table S4.** Primer

| Primer                                     | Sequence (5'->3')                                                    | Restriction site |
|--------------------------------------------|----------------------------------------------------------------------|------------------|
| Su9 <sup>MTS</sup> fwd for pcDNA5/FRT/TO   | GGGGGTACCGCCATGGCCTCCACTCG                                           | KpnI             |
| Tom20 <sup>MTS</sup> fwd for pcDNA5/FRT/TO | GGGGGTACCGCCATGtctagaatgtcccagtc                                     | KpnI             |
| roGFP2-Prx1-NES rev for pcDNA5/FRT/TO      | CCCTCGAGTTAGTCCAGGGTCAGGCG<br>CTCCAGGGGAGGCAGCTGCAGTTTCG<br>ACTTGGTG | XhoI             |
| DAO-FLAG rev for pcDNA5/FRT/TO             | CCCCTCGAGttaCTTGTCGTCATCGTCT<br>TTGTAGTCcagggtcagccgctcc             | XhoI             |
| DAO(no ATG) fwd for pcDNA5/FRT/TO          | GGGGGATCCcacagccagaagag                                              | -                |
| Su9 <sup>MTS</sup> fwd for Piggy Bac       | GGGGCTAGCGCCATGGCCTCCACTCG<br>TGTCC                                  | NheI             |
| DAO-FLAG rev for Piggy Bac                 | CCCGCGGCCGcttaCTTGTCGTCATCG                                          | NotI             |
| PRDX2 fwd for pcDNA5/FRT/TO                | <b>GGAAGCTT</b> GCAGCC <u>ATG</u> GCCTCCGGT<br>AACGCGCGCATCGG        | BamHI            |
| PRDX2-HA rev for pcDNA5/FRT/TO             | CTCGAGCTAagcgtaatctggaacatcgatgggt<br>aATTGTGTTTG                    | HindIII          |

**Appendix Table S5.** CRISPR guides:

| guide                   | Sequence                  |
|-------------------------|---------------------------|
| PRDX1 guide 2 sgRNA fwd | caccgACCATCTGGCATAACAGCTG |
| PRDX1 guide 2 sgRNA rev | aaacCAGCTGTTATGCCAGATGGTc |
| PRDX2 guide 4 sgRNA fwd | caccGTTGCTGAACGCGATGATCT  |
| PRDX2 guide 4 sgRNA rev | aaacAGATCATCGCGTTCAGCAAC  |

**Appendix Table S6.** Number of cells analysed for figures/data

*Statistical analysis:* For Cytation3 measurements, the data was first analyzed for normal distribution using a Shapiro-Wilk-test. As most of the data were not normal distributed, instead of a t-test, a Wilcoxon/Mann-Whitney-U-test was performed and samples were compared in pairs.

| Figure | Cell line         | Medium    | Sensor localization | treatment                     | n   |
|--------|-------------------|-----------|---------------------|-------------------------------|-----|
| 1b     | HEK293 MOCK       | glucose   | cytosol             | H <sub>2</sub> O <sub>2</sub> | 265 |
| 1d     | HEK293 MOCK       | glucose   | IMS                 | AntA                          | 93  |
| 1d     | HEK293 MOCK       | glucose   | IMS                 | Ctr (EtOH)                    | 117 |
| 1d     | HEK293 MOCK       | galactose | IMS                 | AntA                          | 95  |
| 1d     | HEK293 MOCK       | galactose | IMS                 | Ctr (EtOH)                    | 52  |
| 1e     | HEK293 MOCK       | glucose   | matrix              | AntA                          | 133 |
| 1e     | HEK293 MOCK       | glucose   | matrix              | Ctr (EtOH)                    | 135 |
| 1e     | HEK293 MOCK       | galactose | matrix              | AntA                          | 152 |
| 1e     | HEK293 MOCK       | galactose | matrix              | Ctr (EtOH)                    | 125 |
| 1f     | HEK293 MOCK       | glucose   | OMM                 | AntA                          | 142 |
| 1f     | HEK293 MOCK       | glucose   | OMM                 | Ctr (EtOH)                    | 121 |
| 1f     | HEK293 MOCK       | galactose | OMM                 | AntA                          | 96  |
| 1f     | HEK293 MOCK       | galactose | OMM                 | Ctr (EtOH)                    | 72  |
| 1g     | HEK293 MOCK       | glucose   | cytosol             | AntA                          | 82  |
| 1g     | HEK293 MOCK       | glucose   | cytosol             | Ctr (EtOH)                    | 113 |
| 1g     | HEK293 MOCK       | galactose | cytosol             | AntA                          | 144 |
| 1g     | HEK293 MOCK       | galactose | cytosol             | Ctr (EtOH)                    | 94  |
| 1h     | HEK293 MOCK       | galactose | nucleus             | AntA                          | 309 |
| 1h     | HEK293 MOCK       | galactose | nucleus             | Ctr (EtOH)                    | 302 |
| 1h     | HEK293 MOCK       | galactose | PM                  | AntA                          | 236 |
| 1h     | HEK293 MOCK       | galactose | PM                  | Ctr (EtOH)                    | 250 |
|        |                   |           |                     |                               |     |
| 2a     | HEK293 MOCK       | glucose   | matrix              | hypoxia+reoxy                 | 117 |
| 2a     | HEK293 MOCK       | glucose   | matrix              | hypoxia                       | 130 |
| 2a     | HEK293 MOCK       | galactose | matrix              | hypoxia+reoxy                 | 169 |
| 2a     | HEK293 MOCK       | galactose | matrix              | hypoxia                       | 189 |
| 2b     | HEK293 MOCK       | glucose   | IMS                 | hypoxia+reoxy                 | 120 |
| 2b     | HEK293 MOCK       | glucose   | IMS                 | hypoxia                       | 97  |
| 2b     | HEK293 MOCK       | galactose | IMS                 | hypoxia+reoxy                 | 157 |
| 2b     | HEK293 MOCK       | galactose | IMS                 | hypoxia                       | 109 |
| 2c     | HEK293 MOCK       | glucose   | OMM                 | hypoxia+reoxy                 | 110 |
| 2c     | HEK293 MOCK       | glucose   | OMM                 | hypoxia                       | 57  |
| 2c     | HEK293 MOCK       | galactose | OMM                 | hypoxia+reoxy                 | 87  |
| 2c     | HEK293 MOCK       | galactose | OMM                 | hypoxia                       | 56  |
| 2d     | HEK293 MOCK       | glucose   | cytosol             | hypoxia+reoxy                 | 208 |
| 2d     | HEK293 MOCK       | glucose   | cytosol             | hypoxia                       | 143 |
| 2d     | HEK293 MOCK       | galactose | cytosol             | hypoxia+reoxy                 | 242 |
| 2d     | HEK293 MOCK       | galactose | cytosol             | hypoxia                       | 207 |
|        |                   |           |                     |                               |     |
| 3b     | HEK293 matrix-DAO | glucose   | cytosol             | 8 mM L-Ala                    |     |
| 3b     | HEK293 matrix-DAO | glucose   | cytosol             | 1 mM D-Ala                    | 84  |
| 3b     | HEK293 matrix-DAO | glucose   | cytosol             | 2 mM D-Ala                    | 78  |

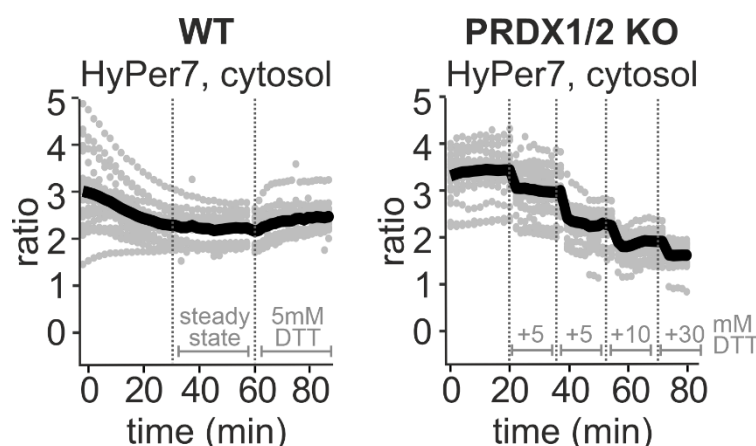
|      |                   |           |             |                               |     |
|------|-------------------|-----------|-------------|-------------------------------|-----|
| 3b   | HEK293 matrix-DAO | glucose   | cytosol     | 4 mM D-Ala                    | 149 |
| 3b   | HEK293 matrix-DAO | glucose   | cytosol     | 8 mM D-Ala                    | 62  |
| 3c   | HEK293 matrix-DAO | glucose   | matrix      | 2 mM D-Ala                    | 107 |
| 3c   | HEK293 matrix-DAO | galactose | matrix      | 2 mM D-Ala                    | 102 |
| 3c   | HEK293 matrix-DAO | glucose   | IMS         | 2 mM D-Ala                    | 107 |
| 3c   | HEK293 matrix-DAO | galactose | IMS         | 2 mM D-Ala                    | 98  |
| 3c   | HEK293 matrix-DAO | glucose   | OMM         | 2 mM D-Ala                    | 182 |
| 3c   | HEK293 matrix-DAO | galactose | OMM         | 2 mM D-Ala                    | 167 |
| 3c   | HEK293 matrix-DAO | glucose   | cytosol     | 2 mM D-Ala                    | 78  |
| 3c   | HEK293 matrix-DAO | galactose | cytosol     | 2 mM D-Ala                    | 128 |
|      |                   |           |             |                               |     |
| 4a   | HEK293 MOCK       | glucose   | matrix      | H <sub>2</sub> O <sub>2</sub> | 228 |
| 4a   | HEK293 MOCK       | galactose | matrix      | H <sub>2</sub> O <sub>2</sub> | 82  |
| 4a   | HEK293 MOCK       | glucose   | IMS         | H <sub>2</sub> O <sub>2</sub> | 91  |
| 4a   | HEK293 MOCK       | galactose | IMS         | H <sub>2</sub> O <sub>2</sub> | 175 |
| 4a   | HEK293 MOCK       | glucose   | OMM         | H <sub>2</sub> O <sub>2</sub> | 49  |
| 4a   | HEK293 MOCK       | galactose | OMM         | H <sub>2</sub> O <sub>2</sub> | 59  |
| 4a   | HEK293 MOCK       | glucose   | cytosol     | H <sub>2</sub> O <sub>2</sub> | 265 |
| 4a   | HEK293 MOCK       | galactose | cytosol     | H <sub>2</sub> O <sub>2</sub> | 93  |
|      |                   |           |             |                               |     |
| 5d   | HEK293 matrix-DAO | galactose | matrix      | 2 mM D-Ala                    | 109 |
| 5d   | HEK293 matrix-DAO | galactose | matrix      | 2mM-D-Ala+Au                  | 130 |
| 5d   | HEK293 matrix-DAO | galactose | cytosol     | 2 mM D-Ala                    | 79  |
| 5d   | HEK293 matrix-DAO | galactose | cytosol     | 2mM-D-Ala+Au                  | 116 |
| 5e   | HEK293 matrix-DAO | galactose | matrix RGP  | 2 mM D-Ala                    | 141 |
| 5e   | HEK293 matrix-DAO | galactose | matrix RGP  | 2mM-D-Ala+Au                  | 241 |
| 5e   | HEK293 matrix-DAO | galactose | cytosol RGP | 2 mM D-Ala                    | 126 |
| 5e   | HEK293 matrix-DAO | galactose | cytosol RGP | 2mM-D-Ala+Au                  | 170 |
|      |                   |           |             |                               |     |
| 6b   | HeLa              | galactose | matrix      | AntA                          | 130 |
| 6b   | HeLa              | galactose | matrix      | Ctr (EtOH)                    | 68  |
| 6b   | HeLa              | galactose | IMS         | AntA                          | 125 |
| 6b   | HeLa              | galactose | IMS         | Ctr (EtOH)                    | 47  |
| 6b   | HeLa              | galactose | OMM         | AntA                          | 130 |
| 6b   | HeLa              | galactose | OMM         | Ctr (EtOH)                    | 49  |
| 6b   | HeLa              | galactose | cytosol     | AntA                          | 204 |
| 6b   | HeLa              | galactose | cytosol     | Ctr (EtOH)                    | 74  |
| 6c   | HeLa matrix-DAO   | glucose   | matrix      | 8 mM D-Ala                    | 111 |
| 6c   | HEK293 matrix-DAO | glucose   | matrix      | 8 mM D-Ala                    | 78  |
| 6c   | HeLa matrix-DAO   | glucose   | IMS         | 8 mM D-Ala                    | 87  |
| 6c   | HEK293 matrix-DAO | glucose   | IMS         | 8 mM D-Ala                    | 54  |
| 6c   | HeLa matrix-DAO   | glucose   | OMM         | 8 mM D-Ala                    | 140 |
| 6c   | HEK293 matrix-DAO | glucose   | OMM         | 8 mM D-Ala                    | 83  |
| 6c   | HeLa matrix-DAO   | glucose   | cytosol     | 8 mM D-Ala                    | 111 |
| 6c   | HEK293 matrix-DAO | glucose   | cytosol     | 8 mM D-Ala                    | 142 |
|      |                   |           |             |                               |     |
| 7c/e | HEK293 MOCK       | glucose   | matrix      | H <sub>2</sub> O <sub>2</sub> | 139 |
| 7c/e | HEK293 PRDX1 KO   | glucose   | matrix      | H <sub>2</sub> O <sub>2</sub> | 164 |
| 7c/e | HEK293 PDRX2 KO   | glucose   | matrix      | H <sub>2</sub> O <sub>2</sub> | 146 |
| 7c/e | HEK293 MOCK       | glucose   | IMS         | H <sub>2</sub> O <sub>2</sub> | 84  |

|      |                            |         |         |                               |     |
|------|----------------------------|---------|---------|-------------------------------|-----|
| 7c/e | HEK293 PRDX1 KO            | glucose | IMS     | H <sub>2</sub> O <sub>2</sub> | 105 |
| 7c/e | HEK293 PDRX2 KO            | glucose | IMS     | H <sub>2</sub> O <sub>2</sub> | 72  |
| 7c/e | HEK293 MOCK                | glucose | OMM     | H <sub>2</sub> O <sub>2</sub> | 68  |
| 7c/e | HEK293 PRDX1 KO            | glucose | OMM     | H <sub>2</sub> O <sub>2</sub> | 151 |
| 7c/e | HEK293 PDRX2 KO            | glucose | OMM     | H <sub>2</sub> O <sub>2</sub> | 113 |
| 7c/e | HEK293 MOCK                | glucose | cytosol | H <sub>2</sub> O <sub>2</sub> | 166 |
| 7c/e | HEK293 PRDX1 KO            | glucose | cytosol | H <sub>2</sub> O <sub>2</sub> | 181 |
| 7c/e | HEK293 PDRX2 KO            | glucose | cytosol | H <sub>2</sub> O <sub>2</sub> | 161 |
| 7d   | HEK293 MOCK                | glucose | matrix  | 4 mM D-Ala                    | 109 |
| 7d   | HEK293 PRDX1 KO            | glucose | matrix  | 4 mM D-Ala                    | 90  |
| 7d   | HEK293 PDRX2 KO            | glucose | matrix  | 4 mM D-Ala                    | 145 |
| 7d   | HEK293 MOCK                | glucose | IMS     | 4 mM D-Ala                    | 92  |
| 7d   | HEK293 PRDX1 KO            | glucose | IMS     | 4 mM D-Ala                    | 65  |
| 7d   | HEK293 PDRX2 KO            | glucose | IMS     | 4 mM D-Ala                    | 92  |
| 7d   | HEK293 MOCK                | glucose | OMM     | 4 mM D-Ala                    | 75  |
| 7d   | HEK293 PRDX1 KO            | glucose | OMM     | 4 mM D-Ala                    | 48  |
| 7d   | HEK293 PDRX2 KO            | glucose | OMM     | 4 mM D-Ala                    | 111 |
| 7d   | HEK293 MOCK                | glucose | cytosol | 4 mM D-Ala                    | 58  |
| 7d   | HEK293 PRDX1 KO            | glucose | cytosol | 4 mM D-Ala                    | 59  |
| 7d   | HEK293 PDRX2 KO            | glucose | cytosol | 4 mM D-Ala                    | 76  |
|      |                            |         |         |                               |     |
| 8d   | HEK293 MOCK                | glucose | IMS     | H <sub>2</sub> O <sub>2</sub> | 84  |
| 8d   | HEK293 PRDX1/2 KO          | glucose | IMS     | H <sub>2</sub> O <sub>2</sub> | 88  |
| 8d   | HEK293 PRDX1/2 KO+PDRX2-HA | glucose | IMS     | H <sub>2</sub> O <sub>2</sub> | 62  |
| 8d   | HEK293 MOCK                | glucose | OMM     | H <sub>2</sub> O <sub>2</sub> | 68  |
| 8d   | HEK293 PRDX1/2 KO          | glucose | OMM     | H <sub>2</sub> O <sub>2</sub> | 134 |
| 8d   | HEK293 PRDX1/2 KO+PDRX2-HA | glucose | OMM     | H <sub>2</sub> O <sub>2</sub> | 108 |
| 8d   | HEK293 MOCK                | glucose | cytosol | H <sub>2</sub> O <sub>2</sub> | 166 |
| 8d   | HEK293 PRDX1/2 KO          | glucose | cytosol | H <sub>2</sub> O <sub>2</sub> | 131 |
| 8d   | HEK293 PRDX1/2 KO+PDRX2-HA | glucose | cytosol | H <sub>2</sub> O <sub>2</sub> | 140 |
| 8e   | HEK293 MOCK                | glucose | Matrix  | 4 mM D-Ala                    | 109 |
| 8e   | HEK293 PRDX1/2 KO          | glucose | Matrix  | 4 mM D-Ala                    | 61  |
| 8e   | HEK293 PRDX1/2 KO+PDRX2-HA | glucose | Matrix  | 4 mM D-Ala                    | 139 |
| 8e   | HEK293 MOCK                | glucose | IMS     | 4 mM D-Ala                    | 92  |
| 8e   | HEK293 PRDX1/2 KO          | glucose | IMS     | 4 mM D-Ala                    | 55  |
| 8e   | HEK293 PRDX1/2 KO+PDRX2-HA | glucose | IMS     | 4 mM D-Ala                    | 83  |
| 8e   | HEK293 MOCK                | glucose | OMM     | 4 mM D-Ala                    | 75  |
| 8e   | HEK293 PRDX1/2 KO          | glucose | OMM     | 4 mM D-Ala                    | 39  |
| 8e   | HEK293 PRDX1/2 KO+PDRX2-HA | glucose | OMM     | 4 mM D-Ala                    | 64  |
| 8e   | HEK293 MOCK                | glucose | Cytosol | 4 mM D-Ala                    | 76  |
| 8e   | HEK293 PRDX1/2 KO          | glucose | cytosol | 4 mM D-Ala                    | 62  |
| 8e   | HEK293 PRDX1/2 KO+PDRX2-HA | glucose | cytosol | 4 mM D-Ala                    | 82  |
| SI1  | HEK293                     | glucose | cytosol | DTT                           | 21  |
| SI1  | HEK293 PRDX1/2 KO          | glucose | cytosol | DTT                           | 22  |

|     |                   |              |         |                 |     |
|-----|-------------------|--------------|---------|-----------------|-----|
| SI3 | HEK293 MOCK       | Glucose      | cytosol | 50 $\mu$ M Rot. | 81  |
| SI3 | HEK293 MOCK       | Glucose      | Matrix  | 50 $\mu$ M Rot. | 103 |
| SI3 | HEK293 MOCK       | Galactose    | cytosol | 50 $\mu$ M Rot. | 83  |
| SI3 | HEK293 MOCK       | Galactose    | Matrix  | 50 $\mu$ M Rot. | 75  |
| SI3 | HEK293 MOCK       | Glucose      | cytosol | Ctrl            | 113 |
| SI3 | HEK293 MOCK       | Glucose      | Matrix  | Ctrl            | 82  |
| SI3 | HEK293 MOCK       | Galactose    | cytosol | Ctrl            | 70  |
| SI3 | HEK293 MOCK       | Galactose    | Matrix  | Ctrl            | 87  |
|     |                   |              |         |                 |     |
| SI4 | HEK293 matrix-DAO | glucose      | nucleus | 2 mM D-Ala      | 133 |
| SI4 | HEK293 matrix-DAO | galactose    | nucleus | 2 mM D-Ala      | 150 |
| SI4 | HEK293 matrix-DAO | glucose      | nucleus | 4 mM D-Ala      | 133 |
| SI4 | HEK293 matrix-DAO | galactose    | nucleus | 4 mM D-Ala      | 150 |
| SI4 | HEK293 matrix-DAO | glucose      | nucleus | 8 mM D-Ala      | 163 |
| SI4 | HEK293 matrix-DAO | galactose    | nucleus | 8 mM D-Ala      | 173 |
| SI4 | HEK293 matrix-DAO | glucose      | PM      | 2 mM D-Ala      | 66  |
| SI4 | HEK293 matrix-DAO | galactose    | PM      | 2 mM D-Ala      | 66  |
| SI4 | HEK293 matrix-DAO | glucose      | PM      | 4 mM D-Ala      | 80  |
| SI4 | HEK293 matrix-DAO | galactose    | PM      | 4 mM D-Ala      | 58  |
| SI4 | HEK293 matrix-DAO | glucose      | PM      | 8 mM D-Ala      | 84  |
| SI4 | HEK293 matrix-DAO | galactose    | PM      | 8 mM D-Ala      | 84  |
|     |                   |              |         |                 |     |
| SI5 | HEK293 MOCK       | galactose    | Cytosol | AntA            | 144 |
| SI5 | HEK293 MOCK       | galactose    | cytosol | Ctrl            | 94  |
| SI5 | HEK293 MOCK       | galactose    | Matrix  | AntA            | 152 |
| SI5 | HEK293 MOCK       | galactose    | matrix  | Ctrl            | 125 |
| SI5 | HeLa              | galactose    | Cytosol | AntA            | 204 |
| SI5 | HeLa              | galactose    | cytosol | Ctrl            | 74  |
| SI5 | HeLa              | galactose    | Matrix  | AntA            | 130 |
| SI5 | HeLa              | galactose    | matrix  | Ctrl            | 68  |
| SI5 | 143b              | galactose    | Cytosol | AntA            | 13  |
| SI5 | 143b              | galactose    | cytosol | Ctrl            | 16  |
| SI5 | 143b              | galactose    | Matrix  | AntA            | 53  |
| SI5 | 143b              | galactose    | matrix  | Ctrl            | 61  |
| SI5 | COS-7             | galactose    | Cytosol | AntA            | 25  |
| SI5 | COS-7             | galactose    | cytosol | Ctrl            | 25  |
| SI5 | COS-7             | galactose    | Matrix  | AntA            | 44  |
| SI5 | COS-7             | galactose    | matrix  | Ctrl            | 17  |
|     |                   |              |         |                 |     |
| SI6 | HEK293 MOCK       | glucose      | matrix  | 2 mM D-Ala      | 107 |
| SI6 | HEK293 MOCK       | glucose      | IMS     | 2 mM D-Ala      | 107 |
| SI6 | HEK293 MOCK       | glucose      | OMM     | 2 mM D-Ala      | 182 |
| SI6 | HEK293 MOCK       | glucose      | cytosol | 2 mM D-Ala      | 78  |
| SI6 | HEK293 MOCK       | glucose      | cytosol | 4 mM D-Ala      | 149 |
| SI6 | HEK293 MOCK       | Shift to gal | matrix  | 2 mM D-Ala      | 120 |
| SI6 | HEK293 MOCK       | Shift to gal | IMS     | 2 mM D-Ala      | 63  |
| SI6 | HEK293 MOCK       | Shift to gal | OMM     | 2 mM D-Ala      | 85  |
| SI6 | HEK293 MOCK       | Shift to gal | cytosol | 2 mM D-Ala      | 98  |
|     |                   |              |         |                 |     |
| SI7 | HEK293 MOCK       | galactose    | matrix  | AntA            | 152 |

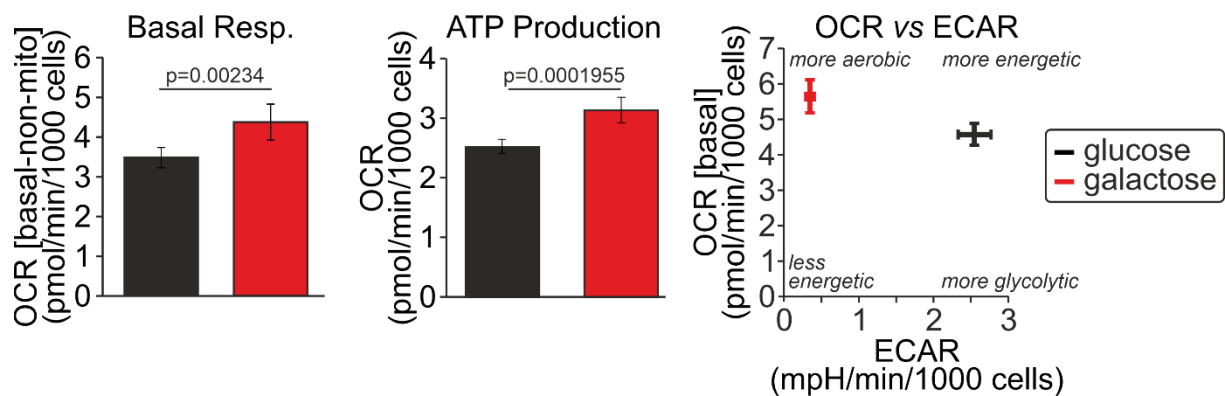
|     |                 |           |         |      |     |
|-----|-----------------|-----------|---------|------|-----|
| SI7 | HEK293 PRDX1 KO | galactose | matrix  | AntA | 159 |
| SI7 | HEK293 PRDX2 KO | galactose | matrix  | AntA | 132 |
| SI7 | HEK293 MOCK     | galactose | IMS     | AntA | 95  |
| SI7 | HEK293 PRDX1 KO | galactose | IMS     | AntA | 118 |
| SI7 | HEK293 PRDX2 KO | galactose | IMS     | AntA | 95  |
| SI7 | HEK293 MOCK     | galactose | OMM     | AntA | 96  |
| SI7 | HEK293 PRDX1 KO | galactose | OMM     | AntA | 83  |
| SI7 | HEK293 PRDX2 KO | galactose | OMM     | AntA | 110 |
| SI7 | HEK293 MOCK     | galactose | cytosol | AntA | 144 |
| SI7 | HEK293 PRDX1 KO | galactose | cytosol | AntA | 204 |
| SI7 | HEK293 PRDX2 KO | galactose | cytosol | AntA | 193 |

## APPENDIX FIGURES

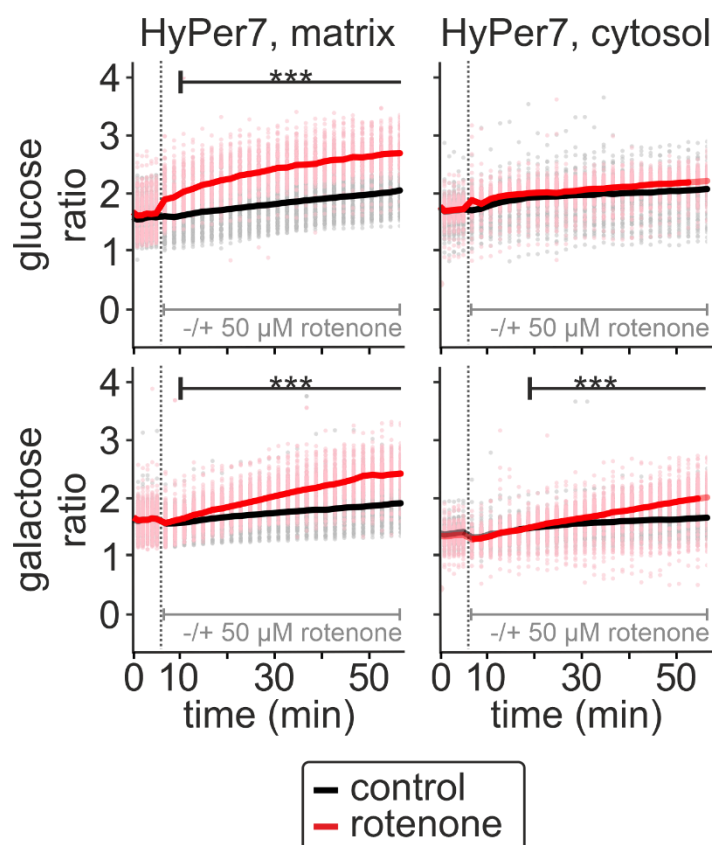


**Appendix Figure S1.** The steady state of the HyPer7 probe. Cells seeded onto 96 well plates and transfected with HyPer7 were grown in medium in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 37°C). Before the measurement, medium was exchanged to minimal medium and cells transferred to the Cytation 3 (5% CO<sub>2</sub>, 37°C). Cells were then left to “adapt” for 30 min. Then, the steady state was monitored. During these 40 min, the cytosolic HyPer7 sensor became progressively more reduced. This is reminiscent of what has been observed in yeast cells for the roGFP2-Tsa2ΔC<sub>R</sub> sensor (Morgan et al, Nature Chem Biol 2016). In the case of this sensor the decrease in probe signal was correlated to decreasing H<sub>2</sub>O<sub>2</sub> generation due to decreasing endogenous oxygen levels after the increased endogenous oxygen levels during the medium exchange. After reaching the steady state, HyPer7 was fully reduced in wild type cells because it could not be reduced any further by addition of DTT. Conversely, in PRDX1/PRDX2 KO cells, the steady state HyPer7 redox state was not fully reduced, and could be further reduced by titrating external DTT to the cells. Solid line represents average, points colored in the lighter version of the respective color are the corresponding ratios measured in individual cells. Cytosolic HyPer7 also reacts towards complex I-generated H<sub>2</sub>O<sub>2</sub> especially if cells were grown on galactose.

The numbers of cells per experiment for each dataset can be found in **Appendix Table S6**.

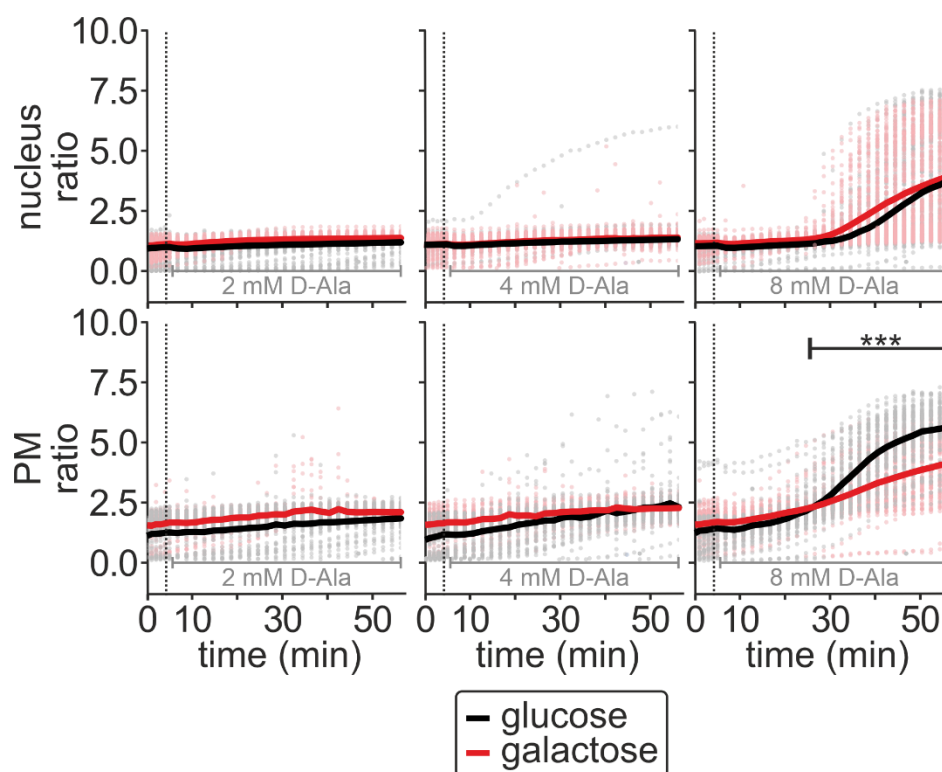


**Appendix Figure S2.** Oxygen Consumption Rate (OCR) measurements were obtained over time using an extracellular flux analyzer (Seahorse Bioscience). The mitochondrial stress test was used to obtain bioenergetics parameters. Basal mitochondrial OCR (*left panel*) was derived by subtracting non-mitochondrial OCR (remaining OCR after antimycin A addition) from basal OCR. ATP-linked OCR (*middle panel*) was derived as the difference between basal and antimycin A inhibited OCR. The bioenergetic profile (*right panel*) was derived by plotting basal ECAR and OCR levels. HEK293 cells grown on glucose displayed high glycolysis and oxidative phosphorylation (highly energetic cells). HEK293 cells grown on galactose displayed high oxidative phosphorylation but comparatively low ECAR classifying them as aerobic. N = 6 (glucose), 5 (galactose); Statistical analysis performed was Student's t-test.



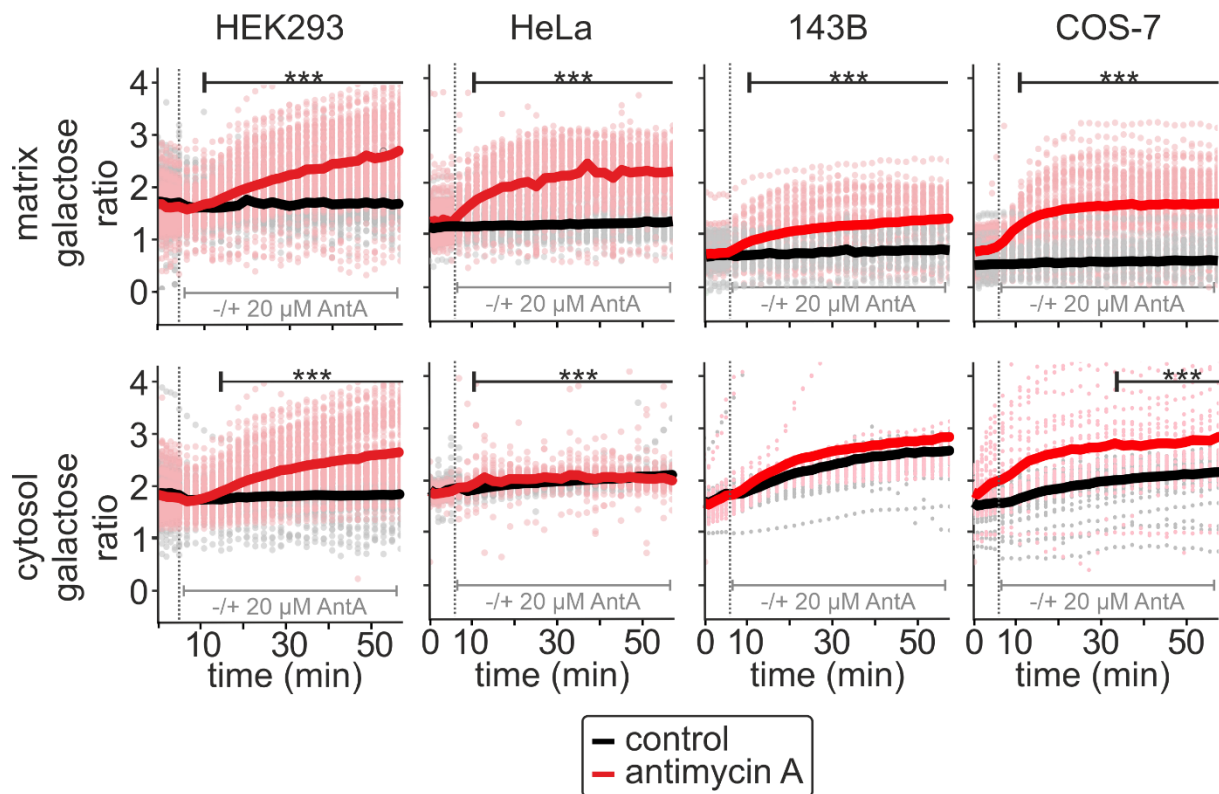
**Appendix Figure S3.** Response of HyPer7 probes targeted to matrix and cytosol to treatment with the complex I inhibitor rotenone. HEK293 cells were grown either with glucose or galactose as carbon source and then treated with rotenone (red curve and data points) or ethanol as control (black curve and data points). Solid line represents average, points colored in the lighter version of the respective color are the corresponding ratios measured in individual cells. Cytosolic HyPer7 also reacts towards complex I-generated  $H_2O_2$  especially if cells were grown on galactose.

The numbers of cells per experiment for each dataset can be found in **Appendix Table S6**. As most of the data were not normal distributed, instead of a t-test, a Wilcoxon/Mann-Whitney-U-test was performed and samples were compared in pairs.   
\*\*\*,  $p \leq 0.001$



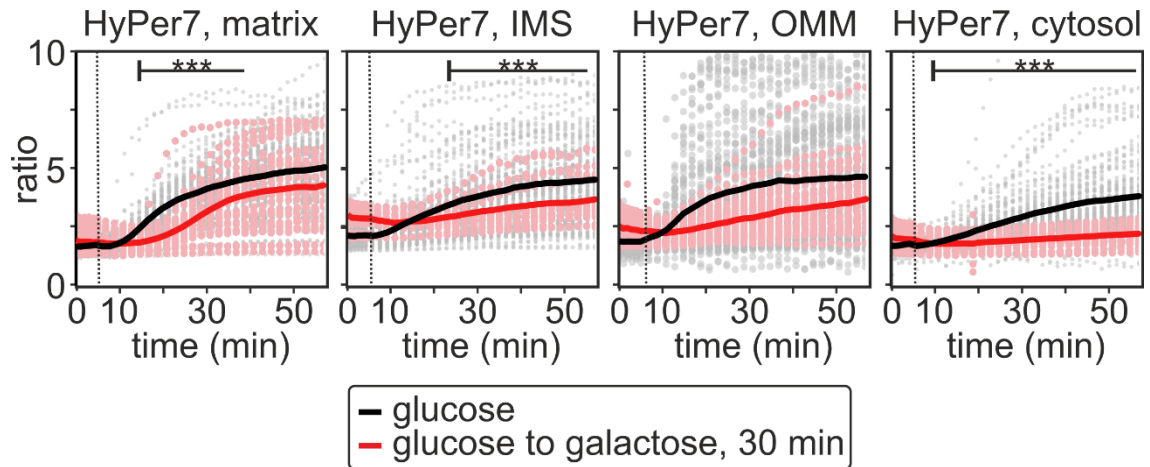
**Appendix Figure S4.** Response of HyPer7 probes targeted to nucleus and plasma membrane (PM) to treatment of mtDAO-expressing HEK293 cells with 2, 4, and 8 mM D-Ala. HEK293 cells were grown either with glucose (black curve and data points) or galactose (red curve and data points) as carbon source. Solid line represents average, points colored in the lighter version of the respective color are the corresponding ratios measured in individual cells. HyPer7 at these locations reacts only to very high concentrations of D-Ala compared to for example OMM- and cytosol-targeted HyPer7.

The numbers of cells per experiment for each dataset can be found in **Appendix Table S6**. As most of the data were not normal distributed, instead of a t-test, a Wilcoxon/Mann-Whitney-U-test was performed and samples were compared in pairs.   
\*\*\*,  $p \leq 0.001$



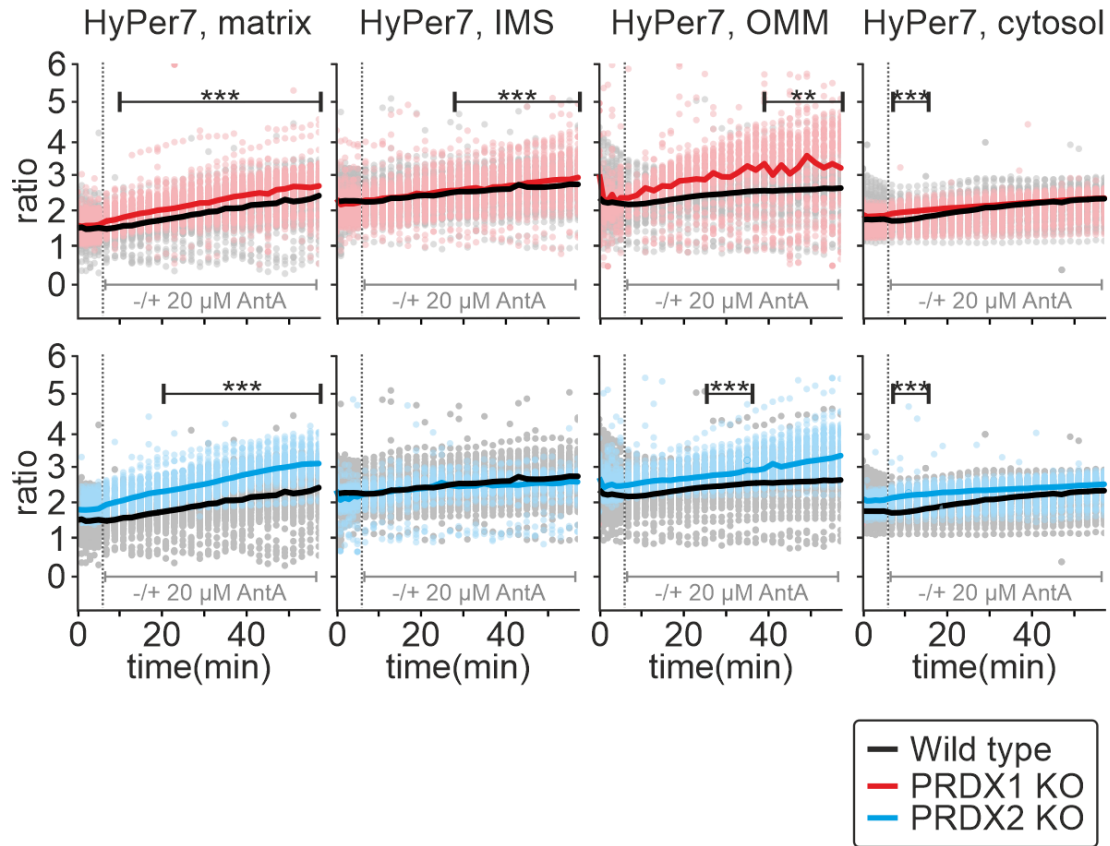
**Appendix Figure S5.** Response of HyPer7 probes targeted to indicated compartments to incubation with antimycin A (AntA, red curve and data points) or ethanol as control (black curve and data points). All cells were grown with galactose as carbon source. Solid line represents average, points colored in the lighter version of the respective color are the corresponding ratios measured in individual cells. While antimycin A-induced mitochondrial  $\text{H}_2\text{O}_2$  production can be robustly monitored in the cytosol of HEK293 and COS-7 cells, this was not the case for 143B and HeLa cells. Data for HEK293 and HeLa cells are presented already in the main manuscript in Figures 1 and 6.

The numbers of cells per experiment for each dataset can be found in **Appendix Table S6**. As most of the data were not normal distributed, instead of a t-test, a Wilcoxon/Mann-Whitney-U-test was performed and samples were compared in pairs. **\*\*\***,  $p \leq 0.001$



**Appendix Figure S6.** Response of HyPer7 probes targeted to indicated compartments to treatment of mtDAO-expressing HEK293 cells with 2 mM D-Ala (cells generated with the Flp-In T-REX-system). HEK293 cells were grown either with glucose as carbon source (black curve and data points) or glucose was replaced by galactose as carbon source 30 min prior to the experiment (red curve and data points). Solid line represents average, points colored in the lighter version of the respective color are the corresponding ratios measured in individual cells. Adaptation to galactose takes place on a short timescale.

The numbers of cells per experiment for each dataset can be found in **Appendix Table S6**. As most of the data were not normal distributed, instead of a t-test, a Wilcoxon/Mann-Whitney-U-test was performed and samples were compared in pairs.\*\*\*,  $p \leq 0.001$



**Appendix Figure S7.** Response of HyPer7 probes targeted to indicated compartments to incubation with antimycin A. Indicated cell lines (black, wild type; red, PRDX1 KO; blue, PRDX2 KO) were grown in galactose-containing medium. Solid line represents average, points coloured in the lighter version of the respective colour are the corresponding ratios measured in individual cells.

The numbers of cells per experiment for each dataset can be found in **Appendix Table S6**. As most of the data were not normal distributed, instead of a t-test, a Wilcoxon/Mann-Whitney-U-test was performed and samples were compared in pairs.

\*\*\*,  $p \leq 0.001$