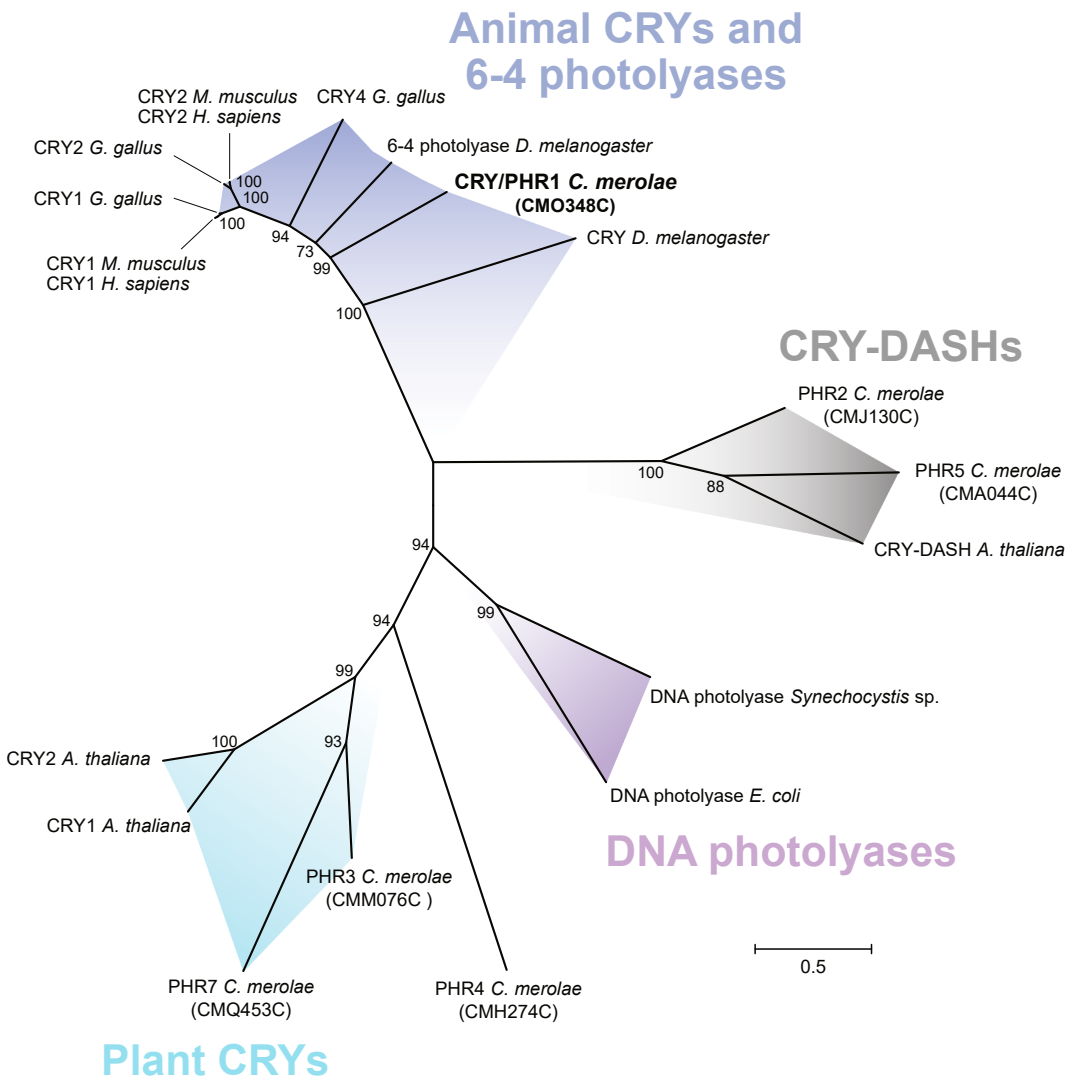


**Fig. S1. Phylogenetic analysis of cryptochromes and photolyases.** The phylogenetic tree was generated using the maximum likelihood method in MEGA X. The subfamilies of animal CRYs and 6-4 photolyases, CRY-DASHs, DNA photolyases, and plant CRYs are shown in blue, black, purple, and light blue, respectively. The numbers at each branch are local bootstrap values estimated using the neighbor-joining method from 1,000 replications.

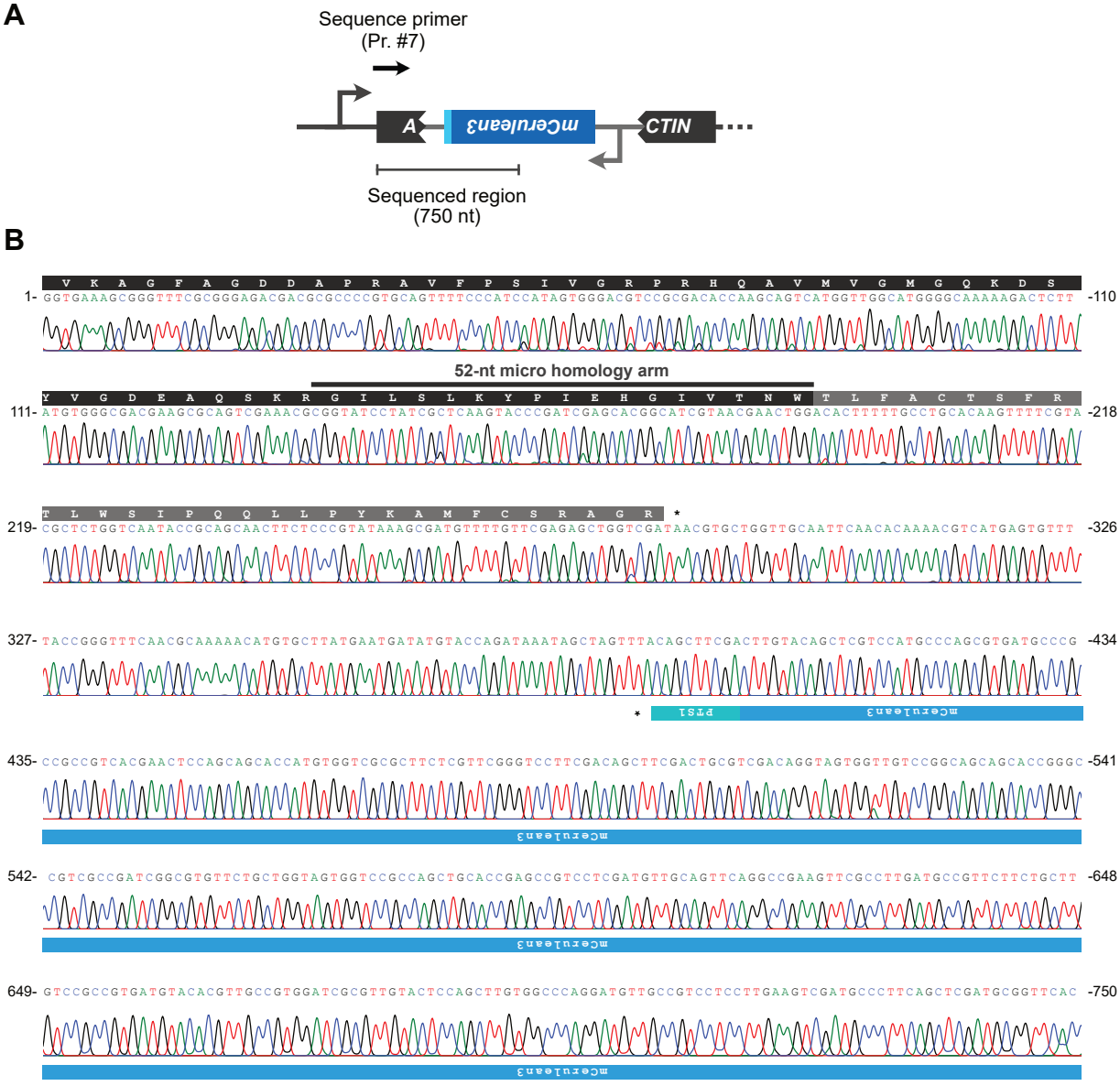


**Fig. S2. DNA sequence at the insertion site of *perCerulean3* knocked into the *ACTIN* locus by CZON-cutter.** (A) Schematic representation of the gene structure in the *ACTIN* knockout (*actin*) strain. (B) Sanger sequencing electropherogram of a transformed strain. The primer sequence (#7) is given in Table S4.

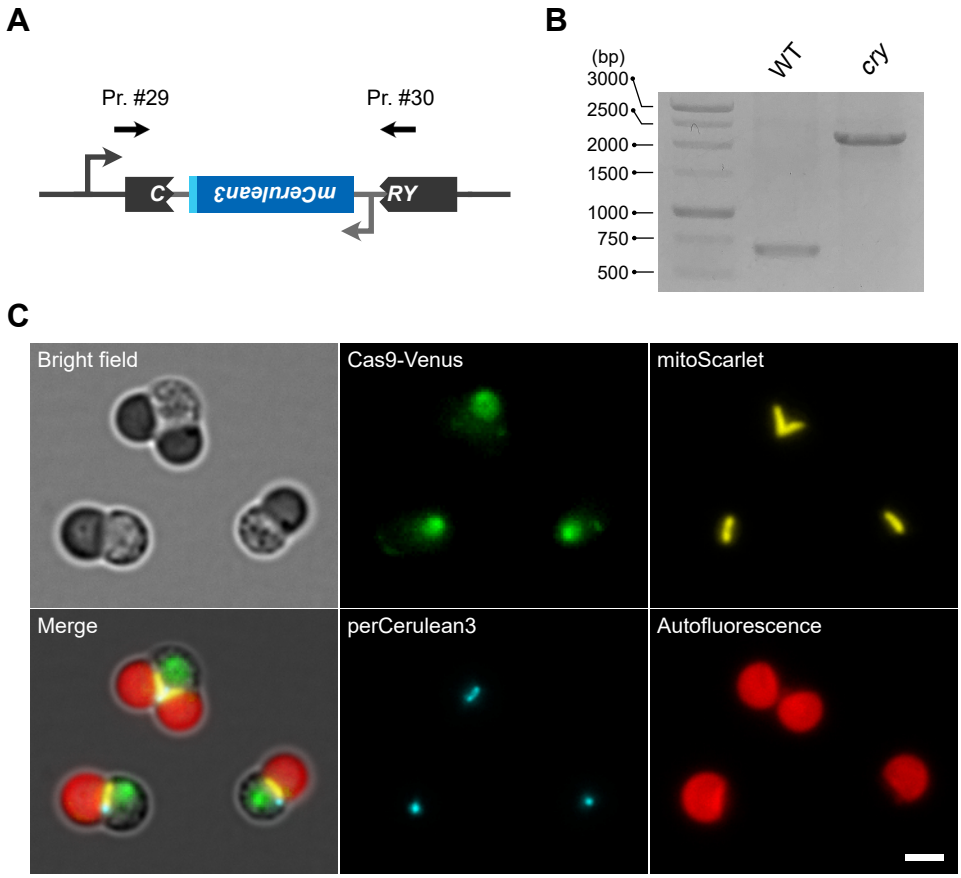


**Fig. S3. Site-specific knock-in of an expression cassette encoding mCerulean3 fused to a peroxisomal targeting signal into the *CRY* locus by CZON-cutter.** (A) Principle behind knocking in a fluorescent reporter expression cassette at the *CRY* locus. (B) Confirmation of the knock-in event at the *CRY* locus by PCR with primer sets #29 and #30 to amplify a fragment of the *CRY* locus. Primer sequences are given in Table S4. (C) Representative images of non-dividing and dividing cells of the *CRY* knockout (*cry*) strain. Green, Cas9-Venus fluorescence; yellow, mitoScarlet fluorescence; blue, perCerulean3 fluorescence; red, chlorophyll autofluorescence.

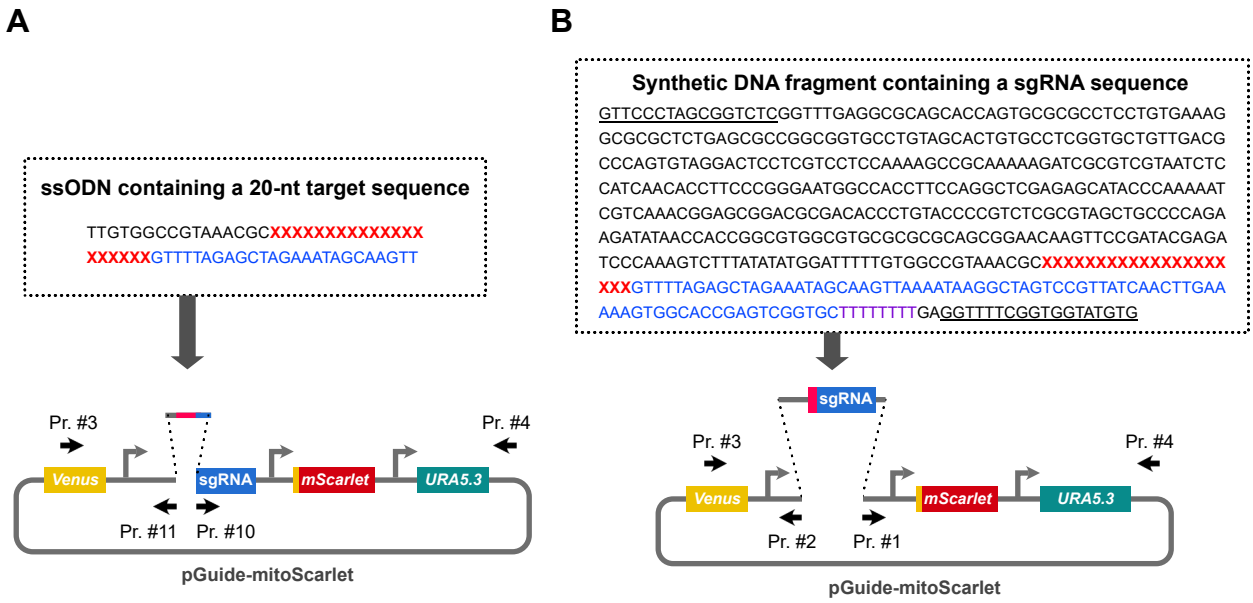


**Fig. S4. Construction of the pGuide-mitoScarlet plasmid targeting a target locus.** For construction of a given single guide RNA (sgRNA), the target sequence can be introduced by following two procedures. (A) A single-stranded oligodeoxynucleotide containing a 20-nt target sequence is combined with a DNA fragment amplified by PCR using primers #10 and #11. (B) A synthetic DNA fragment containing the U6 promoter sequence, a 20-nt target sequence, a sgRNA scaffold sequence, and a transcription termination signal are combined with a DNA fragment amplified by PCR using primers #1 and #2. The 20-nt target sequence is shown as “X” in red. The sgRNA scaffold is shown in blue and the transcription termination signal in purple. Sequences for recombination are underlined. Primer sequences are given in Table S4. See methods for details.

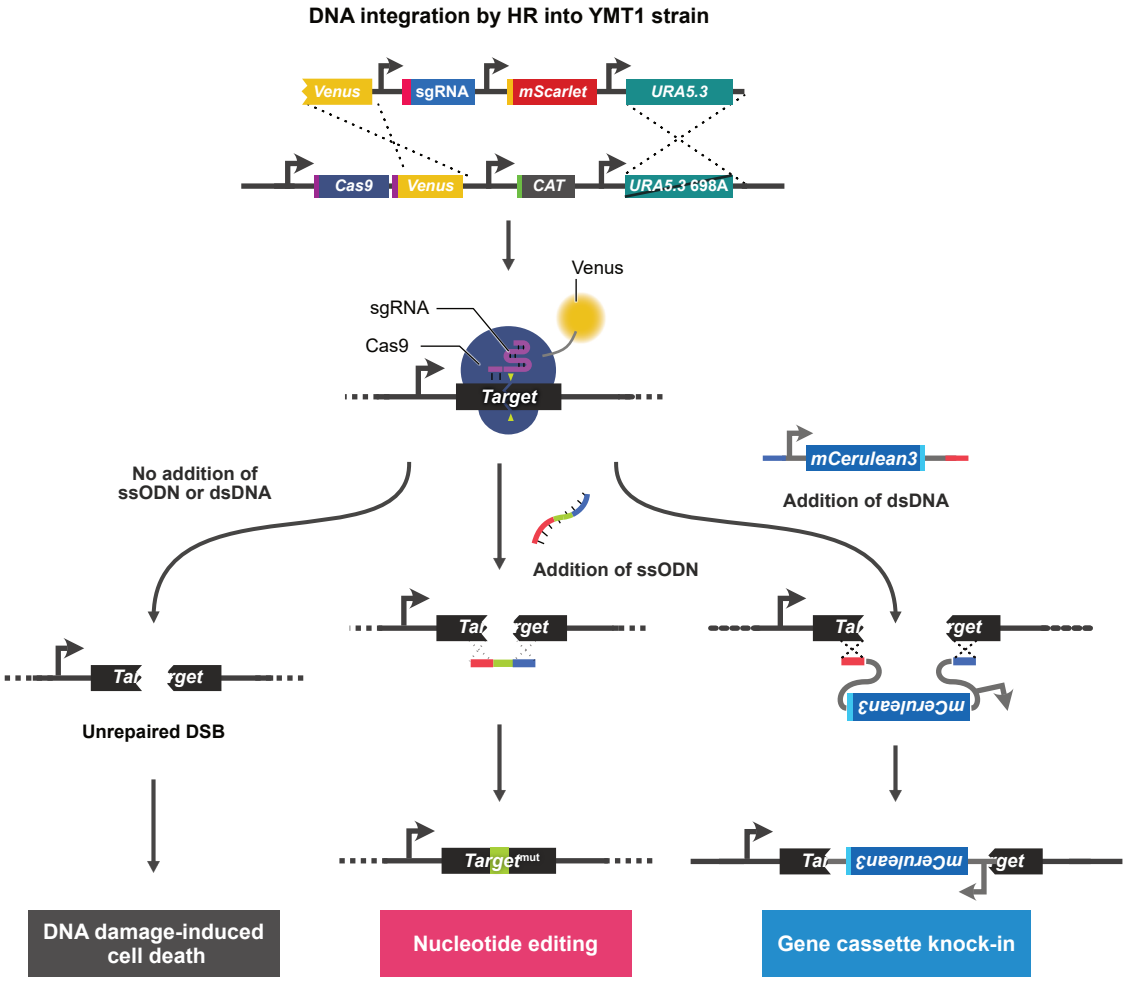


**Fig. S5. Schematic flowchart of CZON-cutter.** For genome editing and gene knock-in, a synthetic DNA fragment containing a single guide RNA (sgRNA) with a specific target sequence was synthesized and then assembled with a linearized pGuide-mitoScarlet vector. A PCR amplicon containing the sgRNA, *mitoScarlet*, and *URA5.3* cassettes was mixed with an 80-nt single-stranded oligodeoxynucleotide (ssODN; for nucleotide modification) or double-stranded DNA (dsDNA; for gene knock-in) and introduced into YMT1 cells. A transformed cell that does not take in the ssODN or dsDNA and does not undergo gene editing will die from DNA damage-induced cell death.

**Table S1.** Summary of datasets for synchronization of the wild-type (WT) and *CRY* knockout (*ko*) strains.

| Strain | Light quality, photon flux density (µmol $m^{-2}$ $s^{-1}$) | Sample number | Number of non-dividing cells | Number of dividing cells | Total number of cells | Percentage of dividing cells | Average (%) | Standard deviation (%) | *p* value (Student's *t*-test) |
|---|---|---|---|---|---|---|---|---|---|
| WT | White<br>Blue: 9.0<br>Green: 15.4<br>Red: 8.8 | 1 | 88 | 95 | 183 | 51.9 | 53.75 | 1.55 | 0.00011 |
| | | 2 | 62 | 78 | 140 | 55.7 | | | |
| | | 3 | 96 | 111 | 207 | 53.6 | | | |
| *ko* | | 1 | 190 | 68 | 258 | 26.4 | 28.2 | 2.4 | |
| | | 2 | 158 | 73 | 231 | 31.6 | | | |
| | | 3 | 181 | 66 | 247 | 26.7 | | | |

| Strain | Light quality, photon flux density (µmol $m^{-2}$ $s^{-1}$) | Sample number | Number of non-dividing cells | Number of dividing cells | Total number of cells | Percentage of dividing cells | Average (%) | Standard deviation (%) | *p* value (Student's *t*-test) |
|---|---|---|---|---|---|---|---|---|---|
| WT | Blue and Red<br>Blue: 15.4<br>Green: 0.8<br>Red: 15.4 | 1 | 179 | 220 | 399 | 55.1 | 53.6 | 1.7 | 0.00078 |
| | | 2 | 115 | 121 | 236 | 51.3 | | | |
| | | 3 | 51 | 61 | 112 | 54.5 | | | |
| *ko* | | 1 | 300 | 228 | 528 | 43.2 | 42.3 | 1.2 | |
| | | 2 | 138 | 94 | 232 | 40.5 | | | |
| | | 3 | 124 | 94 | 218 | 43.1 | | | |

| Strain | Light quality, photon flux density (µmol $m^{-2}$ $s^{-1}$) | Sample number | Number of non-dividing cells | Number of dividing cells | Total number of cells | Percentage of dividing cells | Average (%) | Standard deviation (%) | *p* value (Student's *t*-test) |
|---|---|---|---|---|---|---|---|---|---|
| WT | Red<br>Blue: 0.1<br>Green: 0.2<br>Red: 31.5 | 1 | 152 | 52 | 204 | 25.5 | 22.4 | 2.19 | 0.49304 |
| | | 2 | 123 | 32 | 155 | 20.6 | | | |
| | | 3 | 131 | 35 | 166 | 21.1 | | | |
| *ko* | | 1 | 144 | 22 | 166 | 13.3 | 22.5 | 6.72 | |
| | | 2 | 83 | 28 | 111 | 25.2 | | | |
| | | 3 | 181 | 74 | 255 | 29.0 | | | |

**Table S2.** Top 50 upregulated and downregulated genes between the *CRY* knockout strain and the wi ld-type strain.

**Top 50 upregulated genes**

| # | Gene ID | Annotation | Gene length (bp) | Read count (WT) | Read count (*ko*) | TPM (WT) | TPM (*ko*) | $Log_2$ WT | $Log_2$ KO | Average | LogFC (*ko*/WT) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CMC002C | similar to hedgehog protein | 2,331 | 432 | 1,204 | 133.4 | 357.5 | 7.1 | 8.5 | 7.8 | 1.4 |
| 2 | CMB088C | probable nucleotide binding protein Maf | 621 | 742 | 1,635 | 860.2 | 1,822.5 | 9.7 | 10.8 | 10.3 | 1.1 |
| 3 | CMB086C | hypothetical protein | 540 | 707 | 1,515 | 942.6 | 1,942.1 | 9.9 | 10.9 | 10.4 | 1.0 |
| 4 | CMB085C | hypothetical protein | 2,025 | 296 | 633 | 105.2 | 216.4 | 6.7 | 7.8 | 7.2 | 1.0 |
| 5 | CMB087C | hypothetical protein, conserved | 2,814 | 1,321 | 2,707 | 338.0 | 665.9 | 8.4 | 9.4 | 8.9 | 1.0 |
| 6 | CMB081C | hypothetical protein, conserved | 2,655 | 6,615 | 12,981 | 1793.7 | 3,384.5 | 10.8 | 11.7 | 11.3 | 0.9 |
| 7 | CMB082C | similar to JEMMA protein | 2,202 | 1,975 | 3,845 | 645.7 | 1,208.7 | 9.3 | 10.2 | 9.8 | 0.9 |
| 8 | CMB083C | chaperonin containing TCP1, subunit 3 (gamma) | 1,677 | 1,566 | 2,892 | 672.3 | 1,193.7 | 9.4 | 10.2 | 9.8 | 0.8 |
| 9 | CMD002C | similar to trefoil factor | 3,282 | 145 | 253 | 31.8 | 53.4 | 5.0 | 5.7 | 5.4 | 0.7 |
| 10 | CMW053C | [mt] 30S ribosomal protein S12 | 369 | 450 | 769 | 878.0 | 1,442.6 | 9.8 | 10.5 | 10.1 | 0.7 |
| 11 | CMK045C | pseudouridine synthase 3 | 1,401 | 3,598 | 6,121 | 1848.9 | 3,024.3 | 10.9 | 11.6 | 11.2 | 0.7 |
| 12 | CMI002C | copper-containing amine oxidase | 1,899 | 809 | 1,337 | 306.7 | 487.4 | 8.3 | 8.9 | 8.6 | 0.7 |
| 13 | CMM057C | hypothetical protein | 1,275 | 125 | 205 | 70.6 | 111.3 | 6.1 | 6.8 | 6.5 | 0.7 |
| 14 | CMK046C | uridine 5'-monophosphate synthase (UMP synthase) | 1,389 | 4,028 | 6,569 | 2087.7 | 3,273.7 | 11.0 | 11.7 | 11.4 | 0.6 |
| 15 | CMN328C | probable phosphate/phosphoenolpyruvate translocator precursor | 1,182 | 401 | 641 | 244.2 | 375.4 | 7.9 | 8.6 | 8.2 | 0.6 |
| 16 | CMR293C | hypothetical protein | 690 | 176 | 281 | 183.6 | 281.9 | 7.5 | 8.1 | 7.8 | 0.6 |
| 17 | CMI286C | hypothetical protein | 2,784 | 771 | 1,216 | 199.4 | 302.3 | 7.6 | 8.2 | 7.9 | 0.6 |
| 18 | CML108C | hypothetical protein, conserved | 2,139 | 322 | 505 | 108.4 | 163.4 | 6.8 | 7.4 | 7.1 | 0.6 |
| 19 | CMW007C | [mt] cytochrome B | 1,146 | 770 | 1,196 | 483.7 | 722.4 | 8.9 | 9.5 | 9.2 | 0.6 |
| 20 | CMQ113C | similar to translationally controlled tumor protein (TCTP) (p23) | 507 | 548 | 840 | 778.1 | 1,146.9 | 9.6 | 10.2 | 9.9 | 0.6 |

| # | Gene ID | Annotation | Gene length (bp) | Read count (WT) | Read count (*ko*) | TPM (WT) | TPM (*ko*) | $Log_2$ WT | $Log_2$ KO | Average | LogFC (*ko*/WT) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 21 | CMF130C | hypothetical protein | 1,827 | 261 | 384 | 102.8 | 145.5 | 6.7 | 7.2 | 6.9 | 0.5 |
| 22 | CMT448C | hypothetical protein | 855 | 105 | 154 | 88.4 | 124.7 | 6.5 | 7.0 | 6.7 | 0.5 |
| 23 | CMO348C | Mammalian CRY1 | 1,557 | 734 | 1,076 | 339.4 | 478.4 | 8.4 | 8.9 | 8.7 | 0.5 |
| 24 | CMI306C | mitochondrial ribosomal protein S1 precursor | 1,062 | 2,113 | 3,093 | 1432.4 | 2,016.0 | 10.5 | 11.0 | 10.7 | 0.5 |
| 25 | CMR047C | hypothetical protein | 843 | 272 | 393 | 232.3 | 322.7 | 7.9 | 8.3 | 8.1 | 0.5 |
| 26 | CMS191C | similar to methyltransferase | 654 | 1,964 | 2,793 | 2162.0 | 2,956.2 | 11.1 | 11.5 | 11.3 | 0.5 |
| 27 | CMT597C | heat shock transcription factor | 1,350 | 447 | 634 | 238.4 | 325.1 | 7.9 | 8.3 | 8.1 | 0.4 |
| 28 | CMO066C | cytochrome b6/f complex iron-sulfur subunit precursor | 720 | 433 | 614 | 433.0 | 590.3 | 8.8 | 9.2 | 9.0 | 0.4 |
| 29 | CMP092C | hypothetical protein | 555 | 628 | 879 | 814.6 | 1,096.3 | 9.7 | 10.1 | 9.9 | 0.4 |
| 30 | CMD051C | hypothetical protein | 1,359 | 1,526 | 2,100 | 808.4 | 1,069.7 | 9.7 | 10.1 | 9.9 | 0.4 |
| 31 | CMQ259C | hypothetical protein | 429 | 285 | 391 | 478.3 | 630.9 | 8.9 | 9.3 | 9.1 | 0.4 |
| 32 | CMT349C | hypothetical protein | 336 | 581 | 797 | 1244.9 | 1,642.0 | 10.3 | 10.7 | 10.5 | 0.4 |
| 33 | CMP030C | probable chromatin assembly factor 1 subunit B | 1,569 | 177 | 238 | 81.2 | 105.0 | 6.3 | 6.7 | 6.5 | 0.4 |
| 34 | CMR042C | similar to carbonyl reductase | 855 | 3,999 | 5,375 | 3367.2 | 4,351.7 | 11.7 | 12.1 | 11.9 | 0.4 |
| 35 | CMT300C | similar to origin recognition complex subunit 4 | 1,722 | 240 | 322 | 100.3 | 129.4 | 6.6 | 7.0 | 6.8 | 0.4 |
| 36 | CMT333C | similar to inorganic phosphate transporter | 1,956 | 245 | 328 | 90.2 | 116.1 | 6.5 | 6.9 | 6.7 | 0.4 |
| 37 | CMQ224C | heat shock protein of Hsp90 family | 2,118 | 19,722 | 26,237 | 6703.6 | 8,575.0 | 12.7 | 13.1 | 12.9 | 0.4 |
| 38 | CML030C | DnaJ (Hsp40) homolog, subfamily A | 1,281 | 10,274 | 13,588 | 5774.0 | 7,342.6 | 12.5 | 12.8 | 12.7 | 0.3 |
| 39 | CMT538C | hypothetical protein | 4,101 | 1,260 | 1,663 | 221.2 | 280.7 | 7.8 | 8.1 | 8.0 | 0.3 |
| 40 | CMT049C | hypothetical protein | 1,578 | 479 | 632 | 218.5 | 277.2 | 7.8 | 8.1 | 7.9 | 0.3 |
| 41 | CMR165C | sigma subunit for chloroplast RNA polymerase | 1,947 | 7,267 | 9,586 | 2687.0 | 3,408.1 | 11.4 | 11.7 | 11.6 | 0.3 |
| 42 | CME192C | uridine kinase | 1,284 | 242 | 319 | 135.7 | 172.0 | 7.1 | 7.4 | 7.3 | 0.3 |
| 43 | CMC188C | L-lactate dehydrogenase | 1,062 | 669 | 881 | 453.5 | 574.2 | 8.8 | 9.2 | 9.0 | 0.3 |

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| # | Gene ID | Annotation | Gene length (bp) | Read count (WT) | Read count (*ko*) | TPM (WT) | TPM (*ko*) | $Log_2$ WT | $Log_2$ KO | Average | LogFC (*ko*/WT) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | CME023C | fusion protein of phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazole-succinocarboxamide synthase, chloroplast or mitochondrial precursor | 1,791 | 6,914 | 9,101 | 2779.2 | 3,517.5 | 11.4 | 11.8 | 11.6 | 0.3 |
| 45 | CMA145C | L-lactate dehydrogenase | 1,062 | 2,085 | 2,743 | 1413.4 | 1,787.9 | 10.5 | 10.8 | 10.6 | 0.3 |
| 46 | CMD135C | ATP-binding cassette, sub-family G | 2,475 | 11,268 | 14,696 | 3277.6 | 4,110.3 | 11.7 | 12.0 | 11.8 | 0.3 |
| 47 | CMT087C | similar to DNA replication licensing factor MCM8 | 2,970 | 354 | 461 | 85.8 | 107.4 | 6.4 | 6.7 | 6.6 | 0.3 |
| 48 | CMO111C | outer mitochondrial membrane protein porin | 915 | 1,706 | 2,219 | 1342.3 | 1,678.7 | 10.4 | 10.7 | 10.6 | 0.3 |
| 49 | CMD134C | ATP-binding cassette, sub-family G | 2,286 | 9,095 | 11,824 | 2864.3 | 3,580.4 | 11.5 | 11.8 | 11.6 | 0.3 |
| 50 | CMG131C | hypothetical protein | 966 | 424 | 551 | 316.0 | 394.8 | 8.3 | 8.6 | 8.5 | 0.3 |

**Top 50 downregulated genes**

| # | Gene ID | Annotation | Gene length (bp) | Read count (WT) | Read count (*ko*) | TPM (WT) | TPM (*ko*) | $Log_2$ WT | $Log_2$ *ko* | Average | LogFC (*ko*/WT) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CME121C | probable kynurenine aminotransferase | 1,632 | 1,558 | 720 | 687.3 | 305.4 | 9.4 | 8.3 | 8.8 | –1.2 |
| 2 | CMD163C | probable molybdopterin converting factor small subunit MoaD | 261 | 836 | 579 | 2,306.0 | 1,535.6 | 11.2 | 10.6 | 10.9 | –0.6 |
| 3 | CMP170C | similar to formamidopyrimidine-DNA glycosylase | 780 | 324 | 238 | 299.0 | 211.2 | 8.2 | 7.7 | 8.0 | –0.5 |
| 4 | CMG100C | hypothetical protein | 1,617 | 181 | 134 | 80.6 | 57.4 | 6.3 | 5.8 | 6.1 | –0.5 |
| 5 | CMP240C | retroelement | 372 | 437 | 329 | 845.7 | 612.2 | 9.7 | 9.3 | 9.5 | –0.5 |
| 6 | CMV040C | [pt] ORF105 | 315 | 193 | 147 | 441.1 | 323.0 | 8.8 | 8.3 | 8.6 | –0.4 |
| 7 | CMR137C | similar to developmental gene, multi-sex-combs | 1,530 | 1,381 | 1,060 | 649.8 | 479.6 | 9.3 | 8.9 | 9.1 | –0.4 |
| 8 | CMC047C | hypothetical protein, conserved | 993 | 148 | 114 | 107.3 | 79.5 | 6.7 | 6.3 | 6.5 | –0.4 |
| 9 | CMQ139C | hypothetical protein, conserved | 2,421 | 940 | 725 | 279.5 | 207.3 | 8.1 | 7.7 | 7.9 | –0.4 |
| 10 | CMQ155C | hypothetical protein | 1,518 | 181 | 140 | 85.8 | 63.8 | 6.4 | 6.0 | 6.2 | –0.4 |
| 11 | CMV049C | [pt] ORF73 | 216 | 138 | 107 | 459.9 | 342.9 | 8.8 | 8.4 | 8.6 | –0.4 |
| 12 | CMD073C | hypothetical protein | 357 | 446 | 349 | 899.4 | 676.7 | 9.8 | 9.4 | 9.6 | –0.4 |
| 13 | CMC022C | probable endoplasmic reticulum oxidoreductin 1-Lbeta (ERO1-L) | 1,575 | 1,101 | 862 | 503.3 | 378.9 | 9.0 | 8.6 | 8.8 | –0.4 |
| 14 | CMC142C | hypothetical protein | 993 | 284 | 224 | 205.9 | 156.2 | 7.7 | 7.3 | 7.5 | –0.4 |

| # | Gene ID | Annotation | Gene length (bp) | Read count (WT) | Read count (*ko*) | TPM (WT) | TPM (*ko*) | $Log_2$ WT | $Log_2$ *ko* | Average | LogFC (*ko*/WT) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | CMN138C | probable AAA protein spastin | 1,656 | 887 | 702 | 385.6 | 293.4 | 8.6 | 8.2 | 8.4 | –0.4 |
| 16 | CMT542C | similar to phosphoserine phosphatase | 648 | 315 | 252 | 350.0 | 269.2 | 8.5 | 8.1 | 8.3 | –0.4 |
| 17 | CMF088C | hypothetical protein | 1,143 | 1,473 | 1,182 | 927.8 | 715.8 | 9.9 | 9.5 | 9.7 | –0.4 |
| 18 | CMP321C | similar to anaphase-promoting complex subunit 10 (APC10) | 1,173 | 595 | 478 | 365.2 | 282.1 | 8.5 | 8.1 | 8.3 | –0.4 |
| 19 | CMT450C | similar to nuclear transport factor 2 | 393 | 330 | 266 | 604.5 | 468.5 | 9.2 | 8.9 | 9.1 | –0.4 |
| 20 | CMP182C | hypothetical protein | 1,143 | 875 | 711 | 551.1 | 430.6 | 9.1 | 8.8 | 8.9 | –0.4 |
| 21 | CMR316C | hypothetical protein | 1,131 | 6,860 | 5,606 | 4,366.6 | 3,431.1 | 12.1 | 11.7 | 11.9 | –0.3 |
| 22 | CMP061C | hypothetical protein | 1,008 | 1,159 | 954 | 827.8 | 655.1 | 9.7 | 9.4 | 9.5 | –0.3 |
| 23 | CMP293C | hypothetical protein, conserved | 504 | 356 | 294 | 508.5 | 403.8 | 9.0 | 8.7 | 8.8 | –0.3 |
| 24 | CMN019C | probable inorganic phosphate transporter | 1,914 | 10,345 | 8,553 | 3,891.1 | 3,093.3 | 11.9 | 11.6 | 11.8 | –0.3 |
| 25 | CMC078C | hypothetical protein, conserved | 384 | 371 | 307 | 695.5 | 553.4 | 9.4 | 9.1 | 9.3 | –0.3 |
| 26 | CMT563C | hypothetical protein | 882 | 431 | 357 | 351.8 | 280.2 | 8.5 | 8.1 | 8.3 | –0.3 |
| 27 | CME183C | hypothetical protein | 522 | 10,458 | 8,690 | 14,423.2 | 11,523.8 | 13.8 | 13.5 | 13.7 | –0.3 |
| 28 | CMT031C | hypothetical protein | 663 | 431 | 359 | 468.0 | 374.8 | 8.9 | 8.6 | 8.7 | –0.3 |
| 29 | CMH150C | hypothetical protein, conserved | 744 | 487 | 408 | 471.2 | 379.6 | 8.9 | 8.6 | 8.7 | –0.3 |
| 30 | CMS018C | thioredoxin-like U5 snRNP component dim1 | 423 | 364 | 305 | 619.5 | 499.1 | 9.3 | 9.0 | 9.1 | –0.3 |
| 31 | CMQ121C | hypothetical protein, conserved | 564 | 453 | 380 | 578.2 | 466.4 | 9.2 | 8.9 | 9.0 | –0.3 |
| 32 | CMT114C | similar to ribonuclease PH | 882 | 533 | 448 | 435.1 | 351.6 | 8.8 | 8.5 | 8.6 | –0.3 |
| 33 | CMJ137C | hypothetical protein, conserved | 516 | 201 | 169 | 280.4 | 226.7 | 8.1 | 7.8 | 8.0 | –0.3 |
| 34 | CMV001C | [pt] ABC transporter for iron-sulfur cluster formation (sufB) | 1,395 | 151 | 127 | 77.9 | 63.0 | 6.3 | 6.0 | 6.1 | –0.3 |
| 35 | CMQ466C | similar to 4-aminobutyrate aminotransferase (GABA aminotransferase) | 609 | 120 | 101 | 141.9 | 114.8 | 7.1 | 6.8 | 7.0 | –0.3 |
| 36 | CMO085C | hypothetical protein | 492 | 1,052 | 887 | 1,539.3 | 1,248.0 | 10.6 | 10.3 | 10.4 | –0.3 |
| 37 | CMN051C | similar to 6-pyruvoyltetrahydropterin synthase | 675 | 359 | 303 | 382.9 | 310.7 | 8.6 | 8.3 | 8.4 | –0.3 |
| 38 | CMR072C | hypothetical protein | 360 | 579 | 490 | 1,157.9 | 942.2 | 10.2 | 9.9 | 10.0 | –0.3 |

| # | Gene ID | Annotation | Gene length (bp) | Read count (WT) | Read count (*ko*) | TPM (WT) | TPM (*ko*) | $Log_2$ WT | $Log_2$ *ko* | Average | LogFC (*ko*/WT) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 39 | CML249C | probable 8-oxoguanine-DNA-glycosylase | 1,206 | 3,225 | 2,735 | 1,925.2 | 1,569.8 | 10.9 | 10.6 | 10.8 | –0.3 |
| 40 | CMG014C | similar to G10 protein | 702 | 298 | 253 | 305.6 | 249.5 | 8.3 | 8.0 | 8.1 | –0.3 |
| 41 | CMH224C | mitochondrial ribosomal protein L13 precursor | 678 | 704 | 598 | 747.5 | 610.5 | 9.5 | 9.3 | 9.4 | –0.3 |
| 42 | CMP220C | similar to actin-binding protein, profilin | 591 | 134 | 114 | 163.2 | 133.5 | 7.4 | 7.1 | 7.2 | –0.3 |
| 43 | CMR459C | similar to fructosamine 3 kinase | 924 | 691 | 588 | 538.4 | 440.5 | 9.1 | 8.8 | 8.9 | –0.3 |
| 44 | CMC172C | hypothetical protein, conserved | 1,506 | 1,017 | 868 | 486.2 | 399.0 | 8.9 | 8.6 | 8.8 | –0.3 |
| 45 | CMK039C | photoregulatory zinc-finger protein COP1 | 2,565 | 3,206 | 2,738 | 899.8 | 738.9 | 9.8 | 9.5 | 9.7 | –0.3 |
| 46 | CMT267C | similar to glycosyl transferase | 1,350 | 644 | 550 | 343.4 | 282.0 | 8.4 | 8.1 | 8.3 | –0.3 |
| 47 | CMO069C | hypothetical protein | 399 | 775 | 662 | 1,398.3 | 1,148.5 | 10.4 | 10.2 | 10.3 | –0.3 |
| 48 | CMV089C | [pt] photosystem II protein W | 327 | 151 | 129 | 332.4 | 273.1 | 8.4 | 8.1 | 8.2 | –0.3 |
| 49 | CMP078C | probable beta-galactosidase | 2,331 | 820 | 701 | 253.3 | 208.2 | 8.0 | 7.7 | 7.8 | –0.3 |
| 50 | CMJ262C | MDR1 | 2,100 | 1,429 | 1,223 | 489.9 | 403.1 | 8.9 | 8.7 | 8.8 | –0.3 |

**Table S3**

Evaluation of CRISPR-Cas9 target sites for *CRY*, *ACTIN*, *MDR1*, and *TUBG* by the CRISPRdirect online tool. Target sequences for CRISPR are shown in bold, and PAM sequences are shown in italics.

| Target gene | Start | End | Strand | Sequence | GC (%) | Tm (ºC) | TTTT | Hit_20mer | Hit_12mer | Hit_8mer |
|---|---|---|---|---|---|---|---|---|---|---|
| CMO348C *CRY* | 232 | 254 | + | **tctagactgtttgtgcttcg***ggg* | 45 | 68.62 | 0 | 1 | 1 | 116 |
| CMM237C *ACTIN* | 239 | 261 | + | **gggatgatatggaaaag atc***tgg* | 40 | 65.77 | 0 | 1 | 1 | 28 |
| CMJ262C *MDR1* | 254 | 276 | – | **gggctcgtgggaaagta cgg***cgg* | 65 | 79.45 | 0 | 1 | 1 | 19 |
| CMN304C *TUBG* | 863 | 885 | + | **cgtacagccaccaagat ctg***cgg* | 55 | 73.39 | 0 | 1 | 1 | 18 |

**Table S4.** Primers and synthetic DNA fragments used in this study. Sequences for recombination are underlined. An additional nucleotide and inserted stop codons (TGA) are shown in bold. The complete nucleotide sequence of the synthetic DNA fragments for single-guide RNAs (sgRNAs) targeting *CRY*, *ACTIN*, and *MDR1* are listed as #16 to #18. Target sequences are shown in red. sgRNA scaffold sequences and transcription termination signals are shown in blue and purple, respectively.

| DNA # | Sequence | Description |
|---|---|---|
| 1 | GGTTTTCGGTGGTATGTGATTTACAGCGAAAC | For PCR amplification of pGuide-mitoScarlet vector to assemble with a synthetic DNA |
| 2 | GAGACCGCTAGGGAACGTTCGG | |
| 3 | CGGCGATGTCAACGGTCACAAATTCTC | For transformation of DNA fragments containing sgRNA, *mitoScarlet*, and *URA5.3* gene cassettes |
| 4 | AGCAGCTGACTGTATCTCTATTCTTAGGAATCC | |
| 5 | GGATGATCCTCGGGGGAGATGCGCAACTCGTTGTAAAAAGTGT GGTACCACGACGAGAACGTATAAGGAGTGCG | For PCR amplification of the *perCerulean3* gene cassette for the knock-in in the *ACTIN* locus |
| 6 | CGGTATCCTATCGCTCAAGTACCCGATCGAGCACGGCATCGTA ACGAACTGGACACTTTTTGCCTGCACAAGTTTTCG | |
| 7 | ATGACAGAGGAGGAAATAACAGCTCTTGTG | For PCR amplification of *ACTIN* ORF |
| 8 | GAAACATTTGCGGTGTACCACAGAG | |
| 9 | TTGTGGCCGTAAACGC**G***CGTACAGCCACCAAGATCTG*GTTTTA GAGCTAGAAATAGCAAGTT | For construction of pGuide-*TUBG*$_{863\text{-}885}$-mitoScarlet |
| 10 | GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAG | For PCR amplification of pGuide-mitoScarlet vector to assemble with a ssODN |
| 11 | GCGTTTACGGCCACAAAAATCCATATATAAAGAC | |
| 12 | AGGAGTTCCACTCGGGAGCTTTTCTTGAGAGCTTTGACAACGAA GGCGTC**TGA**ACACTTTTTGCCTGCACAAGTTTTCG | For PCR amplification of the *perCerulean3* gene cassette for the knock-in in the *MDR1* locus |
| 13 | TCAGTATACGAGTGACGTAGGCTTTCGTCGCTGTTCCACGAGC GGGCTCGCGACGAGAACGTATAAGGAGTGCG | |
| 14 | CGATGGTCATGGCAGCGTCGACCACGACGATGCGATTCCCAGC GTACAGC**TGA**ACACTTTTTGCCTGCACAAGTTTTCG | For PCR amplification of the *perCerulean3* gene cassette for the knock-in in the *TUBG* locus |
| 15 | CTTGCCACCAGAAAGTGACAAGGCGGGAGCGGCACCAATGTAG ACATCAGCGACGAGAACGTATAAGGAGTGCG | |
| 16 | GTTCCCTAGCGGTCTCGGTTTGAGGCGCAGCACCAGTGCGCGC CTCCTGTGAAAGGCGCGCTCTGAGCGCCGGCGGTGCCTGTAG CACTGTGCCTCGGTGCTGTTGACGCCCAGTGTAGGACTCCTCG TCCTCCAAAAGCCGCAAAAAGATCGCGTCGTAATCTCCATCAAC ACCTTCCCGGGAATGGCCACCTTCCAGGCTCGAGAGCATACCC AAAAATCGTCAAACGGAGCGGACGCGACACCCTGTACCCCGTC TCGCGTAGCTGCCCCAGAAGATATAACCACCGGCGTGGCGTGC GCGCGCAGCGGAACAAGTTCCGATACGAGATCCCAAAGTCTTTA TATATGGATTTTTGTGGCCGTAAACGCGTCTAGACTGTTTGTGCT TCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG TTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGAG GTTTTCGGTGGTATGTG | Synthetic DNA for sgRNA targeting 232–254 bp of the *CRY* locus |
| 17 | GTTCCCTAGCGGTCTCGGTTTGAGGCGCAGCACCAGTGCGCGC CTCCTGTGAAAGGCGCGCTCTGAGCGCCGGCGGTGCCTGTAG CACTGTGCCTCGGTGCTGTTGACGCCCAGTGTAGGACTCCTCG TCCTCCAAAAGCCGCAAAAAGATCGCGTCGTAATCTCCATCAAC ACCTTCCCGGGAATGGCCACCTTCCAGGCTCGAGAGCATACCC AAAAATCGTCAAACGGAGCGGACGCGACACCCTGTACCCCGTC TCGCGTAGCTGCCCCAGAAGATATAACCACCGGCGTGGCGTGC GCGCGCAGCGGAACAAGTTCCGATACGAGATCCCAAAGTCTTTA TATATGGATTTTTGTGGCCGTAAACGCGGGATGATATGGAAAAGA TCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGT TATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGAG GTTTTCGGTGGTATGTG | Synthetic DNA for sgRNA targeting 239–261 bp of the *ACTIN* locus |

| DNA # | Sequence | Description |
|---|---|---|
| 18 | GTTCCCTAGCGGTCTCGGTTTGAGGCGCAGCACCAGTGCGCGC CTCCTGTGAAAGGCGCGCTCTGAGCGCCGGCGGTGCCTGTAG CACTGTGCCTCGGTGCTGTTGACGCCCAGTGTAGGACTCCTCG TCCTCCAAAAGCCGCAAAAAGATCGCGTCGTAATCTCCATCAAC ACCTTCCCGGGAATGGCCACCTTCCAGGCTCGAGAGCATACCC AAAAATCGTCAAACGGAGCGGACGCGACACCCTGTACCCCGTC TCGCGTAGCTGCCCCAGAAGATATAACCACCGGCGTGGCGTGC GCGCGCAGCGGAACAAGTTCCGATACGAGATCCCAAAGTCTTTA TATATGGATTTTTGTGGCCGTAAACGCGGGCTCGTGGGAAAGTA CGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG TTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGAG GTTTTCGGTGGTATGTG | Synthetic DNA for sgRNA targeting 254–276 bp of the *MDR1* locus |
| 19 | GTTTCTTCGTTCGTTGACCATGGTGTCGAAGGGCGAGGAGC | For PCR amplification of *perCerulean3* ORF |
| 20 | GCTAGTTTACAGCTTCGACTTGTACAGCTCGTCCATGCCCAG | |
| 21 | ATTGACGCGTATTGGGATCGACGAGAACGTATAAGGAGTGCG | For PCR amplification of a *ApcC* promoter sequence |
| 22 | GGTCAACGAACGAAGAAACACAGAGAACAAAG | |
| 23 | TCGAAGCTGTAAACTAGCTATTTATCTGGTACATATCATTCATAA GCAC | For PCR amplification of a *TUBB* 3′ UTR |
| 24 | CGCGCCATTGGGATACACTTTTTGCCTGCACAAGTTTTCG | |
| 25 | ATCCCAATGGCGCGCCGAGCTTG | For PCR amplification of pUC57 vector DNA |
| 26 | ATCCCAATACGCGTCAATTCACTGGC | |
| 27 | TTCTGCTAGAGTGCTTACAGGATCTGGACCAGCAACTTCGCAAG CTG**TGA**ACACTTTTTGCCTGCACAAGTTTTCG | For PCR amplification of the *perCerulean3* gene cassette for the knock-in in the *CRY* locus |
| 28 | GCGTATGATACTTTCGGAAAAAGACGGGTAGTTGCTCCAGCGG ATTGCCCGACGAGAACGTATAAGGAGTGCG | |
| 29 | ATGTGGGTCATCTTGACCATGGCAAG | For PCR amplification of *CRY* ORF |
| 30 | CGTAAAAAGTTCTCCATGCGCCTGAG | |

ORF, open reading frame; ssODN, single-stranded oligodeoxynucleotide; UTR, untranslated region.

**Table S5.** Complete amino acid sequence of Cas9-Venus. *Streptococcus pyogenes* Cas9 and Venus are shown in blue and orange, respectively. The nuclear localization sequences are shown in purple and bold font, the 3× FLAG tag in green, and the 3× HA tag in red and italics.

**Cas9-Venus (1,689 aa)**

MDYKDHDGDYKDHDIDYKDDDDKMA**PKKKRKV**GIHGVPAADKKYSIGLDIGTNSVGWAVI
TDEYKVPSKKFKVLGNTDRHSIKKNLIGALLFDSGETAEATRLKRTARRRYTRRKNRICYLQ
EIFSNEMAKVDDSFFHRLEESFLVEEDKKHERHPIFGNIVDEVAYHEKYPTIYHLRKKLVDST
DKADLRLIYLALAHMIKFRGHFLIEGDLNPDNSDVDKLFIQLVQTYNQLFEENPINASGVDAK
AILSARLSKSRRLENLIAQLPGEKKNGLFGNLIALSLGLTPNFKSNFDLAEDAKLQLSKDTYD
DDLDNLLAQIGDQYADLFLAAKNLSDAILLSDILRVNTEITKAPLSASMIKRYDEHHQDLTLLK
ALVRQQLPEKYKEIFFDQSKNGYAGYIDGGASQEEFYKFIKPILEKMDGTEELLVKLNREDL
LRKQRTFDNGSIPHQIHLGELHAILRRQEDFYPFLKDNREKIEKILTFRIPYYVGPLARGNSR
FAWMTRKSEETITPWNFEEVVDKGASAQSFIERMTNFDKNLPNEKVLPKHSLLYEYFTVYN
ELTKVKYVTEGMRKPAFLSGEQKKAIVDLLFKTNRKVTVKQLKEDYFKKIECFDSVEISGVE
DRFNASLGTYHDLLKIIKDKDFLDNEENEDILEDIVLTLTLFEDREMIEERLKTYAHLFDDKVM
KQLKRRRYTGWGRLSRKLINGIRDKQSGKTILDFLKSDGFANRNFMQLIHDDSLTFKEDIQK
AQVSGQGDSLHEHIANLAGSPAIKKGILQTVKVVDELVKVMGRHKPENIVIEMARENQTTQ
KGQKNSRERMKRIEEGIKELGSQILKEHPVENTQLQNEKLYLYYLQNGRDMYVDQELDINR
LSDYDVDHIVPQSFLKDDSIDNKVLTRSDKNRGKSDNVPSEEVVKKMKNYWRQLLNAKLIT
QRKFDNLTKAERGGLSELDKAGFIKRQLVETRQITKHVAQILDSRMNTKYDENDKLIREVKV
ITLKSKLVSDFRKDFQFYKVREINNYHHAHDAYLNAVVGTALIKKYPKLESEFVYGDYKVYD
VRKMIAKSEQEIGKATAKYFFYSNIMNFFKTEITLANGEIRKRPLIETNGETGEIVWDKGRDF
ATVRKVLSMPQVNIVKKTEVQTGGFSKESILPKRNSDKLIARKKDWDPKKYGGFDSPTVAY
SVLVVAKVEKGKSKKLKSVKELLGITIMERSSFEKNPIDFLEAKGYKEVKKDLIIKLPKYSLFE
LENGRKRMLASAGELQKGNELALPSKYVNFLYLASHYEKLKGSPEDNEQKQLFVEQHKHY
LDEIIEQISEFSKRVILADANLDKVLSAYNKHRDKPIREQAENIIHLFTLTNLGAPAAFKYFDTT
IDRKRYTSTKEVLDATLIHQSITGLYETRIDLSQLGGDEGA**PKKKRKV**GSSVVSKGEELFTG
VVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVTTLGYGLQCFA
RYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKE
DGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPV
LLPDNHYLSYQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK*MYPYDVPDYAGYPYD*
*VPDYAGYPYDVPDYA*

**Table S6.** Complete nucleotide sequence of *Cyanidioschyzon merolae* codon-optimized *mCerulean3* with the sequence for a peroxisomal targeting signal 1 (PTS1). The PTS1 sequence is shown in blue.

ATGGTGTCGAAGGGCGAGGAGCTGTTCACGGGCGTGGTGCCGATCCTGGTGGAGCT
GGACGGCGACGTGAACGGCCACAAGTTCTCGGTGTCGGGTGAGGGCGAGGGTGACG
CGACGTACGGCAAGCTGACGCTGAAGTTCATCTGCACGACGGGCAAGCTGCCGGTGC
CGTGGCCGACGCTGGTGACGACGCTGTCGTGGGGCGTGCAGTGCTTCGCGCGCTAC
CCGGACCACATGAAGCAGCACGACTTCTTCAAGTCGGCGATGCCGGAGGGCTACGTG
CAGGAGCGCACGATCTTCTTCAAGGACGACGGCAACTACAAGACGCGCGCGGAGGTG
AAGTTCGAGGGCGACACGCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAG
GAGGACGGCAACATCCTGGGCCACAAGCTGGAGTACAACGCGATCCACGGCAACGTG
TACATCACGGCGGACAAGCAGAAGAACGGCATCAAGGCGAACTTCGGCCTGAACTGC
AACATCGAGGACGGCTCGGTGCAGCTGGCGGACCACTACCAGCAGAACACGCCGATC
GGCGACGGCCCGGTGCTGCTGCCGGACAACCACTACCTGTCGACGCAGTCGAAGCT
GTCGAAGGACCCGAACGAGAAGCGCGACCACATGGTGCTGCTGGAGTTCGTGACGG
CGGCGGGCATCACGCTGGGCATGGACGAGCTGTACAAGTCGAAGCTG