# Title: Open and closed evolutionary paths for drastic morphological changes, involving serial gene duplication, sub-functionalization, and selection

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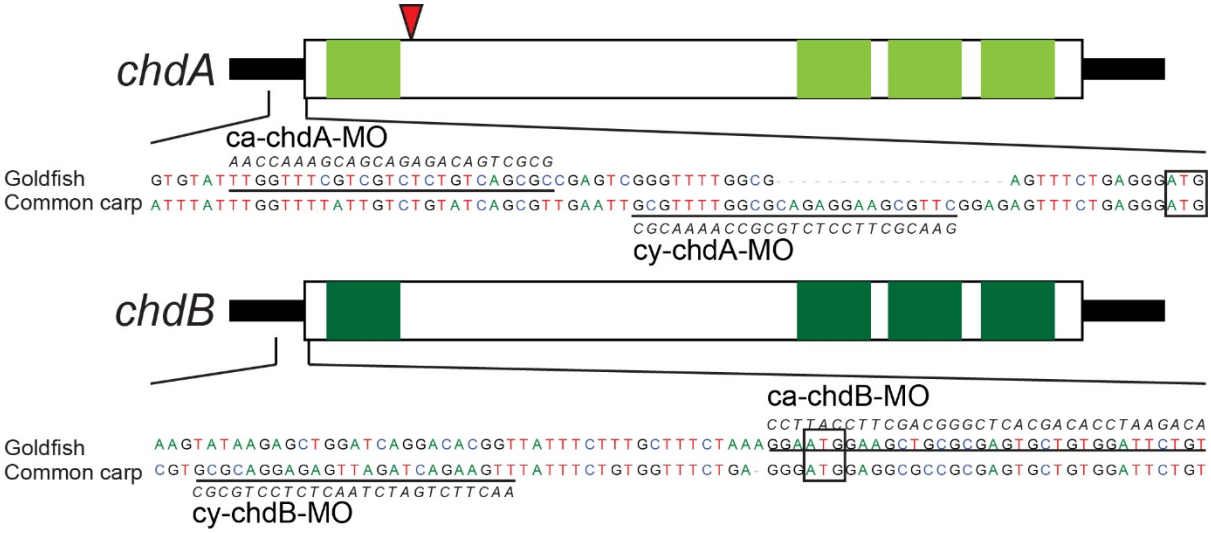
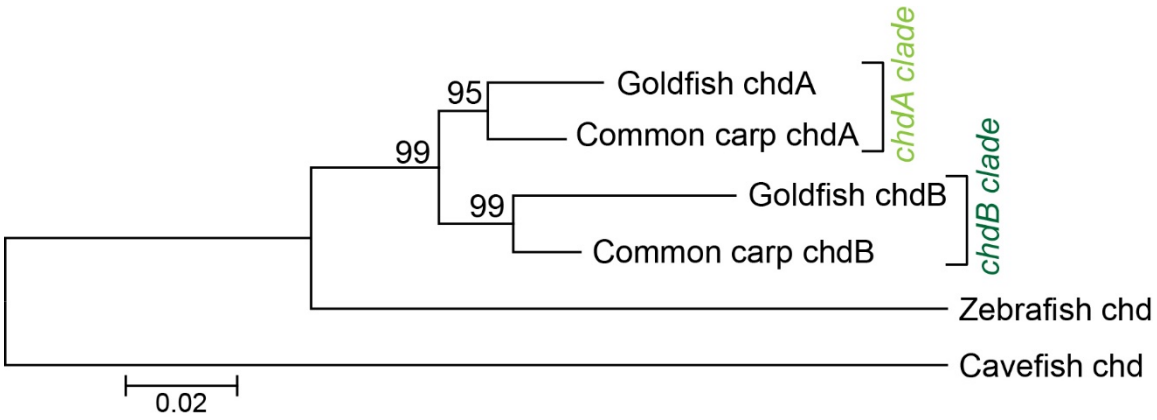
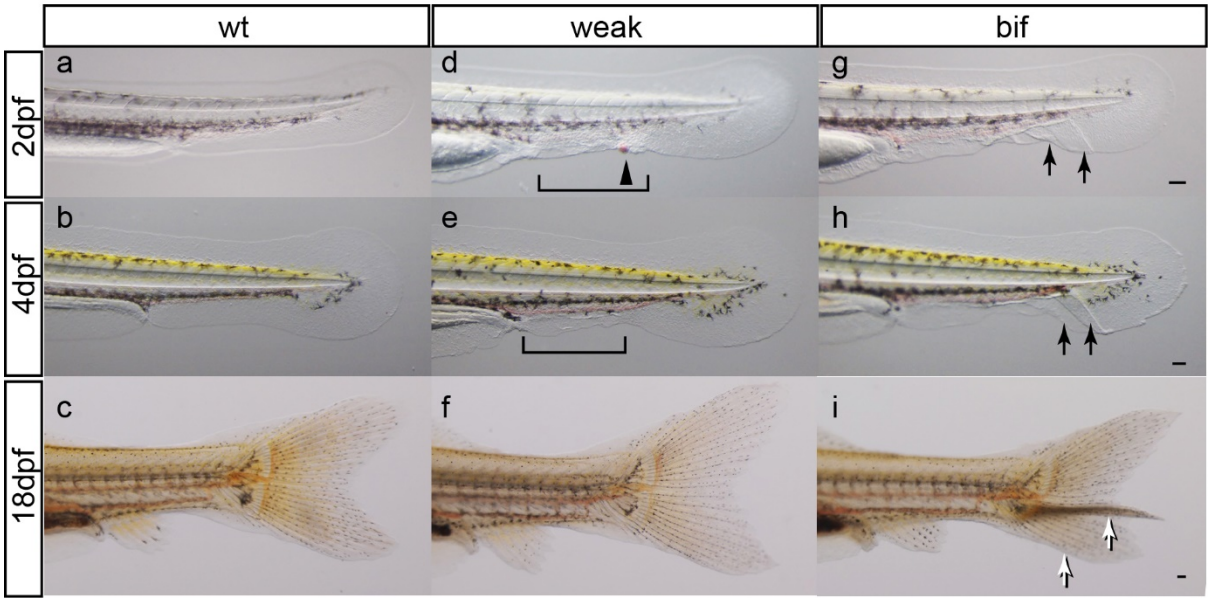


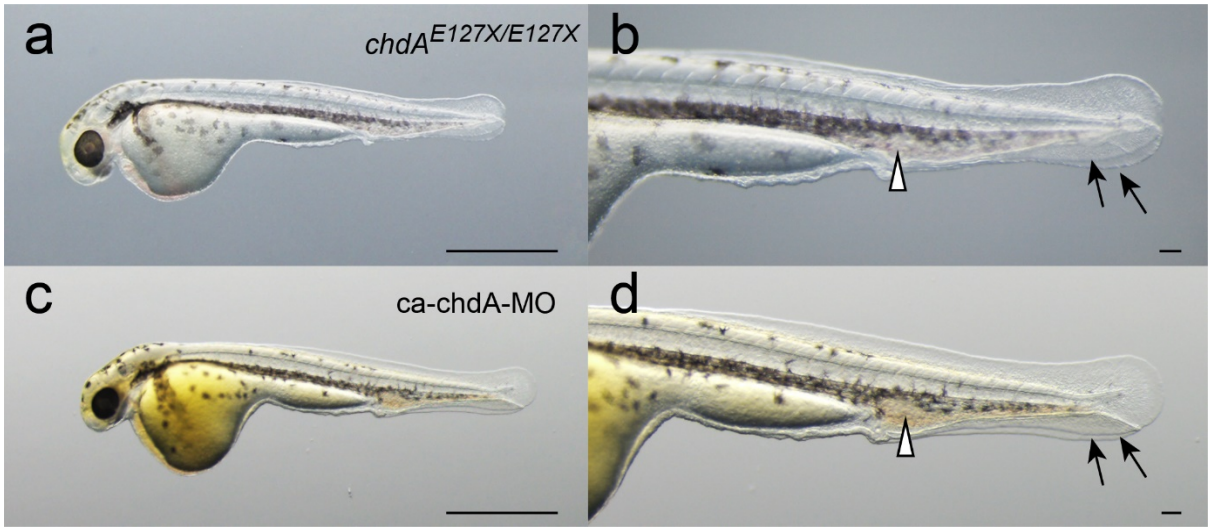

**Supplementary Figure S1: Schematic views of ChdA and -B proteins and partial sequences of *chdA* and *–B* mRNA with morpholino-binding regions.** The positions of cysteine-rich domains are indicated by light green (*chdA*) and dark green (*chdB*) boxes. The red arrowhead indicates the position of the stop codon mutation (*chdA*$^{E127X}$ allele) reported in twin-tail goldfish (Abe et al., 2014). Four morpholino oligonucleotides (ca-chdA-MO, ca-chdB-MO, cy-chdA-MO, and cy-chdB-MO) were designed for blocking the translation of goldfish (ca) or common carp (cy) *chdA* and *-B* genes.




**Supplementary Figure S2: Phylogenetic relationship of *chordin* genes.** A phylogenetic tree of the *chordin* genes from goldfish, common carp, zebrafish, and cavefish was reconstructed by the maximum likelihood method based on a multiple alignment of amino acid sequences, using the MEGA5 program. The phylogenetic relationship between *chdA* and *–B* clades was clearly separated with high support values (over 95%). Chordin sequence accession numbers from DDBJ/EMBL/NCBI and transcript IDs from Ensemble Genome Browser are as follows: Zebrafish chordin, NM_130973; Cavefish chordin, ENSAMXT00000012078; Goldfish chdA, BAO51895.1; Goldfish chdB, BAO51897; Common carp chdA, LC092194; Common carp chdB LC092195. The final dataset contained a total of 957 positions.

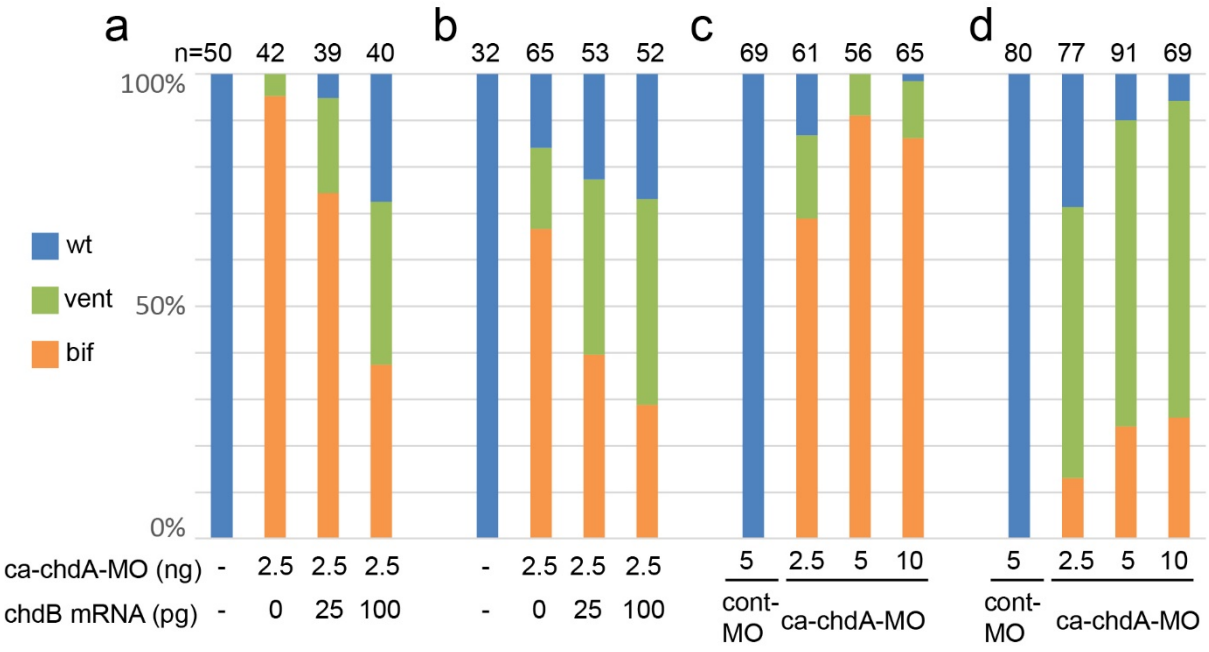



**Supplementary Figure S3: Post-hatching development of the goldfish caudal region.** (**a-c**) Wildtype (single-tail) goldfish individual. (**d-f**) Weakly ventralized goldfish individual bearing the *chdA*$^{E127X/E127X}$ locus. (**g-i**) Bifurcated caudal fin individual bearing the *chdA*$^{E127X/E127X}$ locus. The development of caudal regions was traced in identical individuals at 2, 4, and 18 days post-fertilization (dpf). Black arrows, white arrows, black arrowheads, and brackets indicate bifurcated fin folds, bifurcated caudal fins, ectopic accumulation of blood cells, and fin fold areas showing developmental malformations, respectively. In the weakly ventralized individual, the malformed area and ectopic accumulation of blood cells ultimately reduced in size and disappeared, while the bifurcated fin fold finally differentiated into a bifurcated caudal fin, at 18 dpf. Scale bar = 0.1mm. Upper (**a**, **d**, **g**), middle (**b**, **e**, **h**), and lower (**c**, **f**, **i**) panels are all presented at the same magnification.

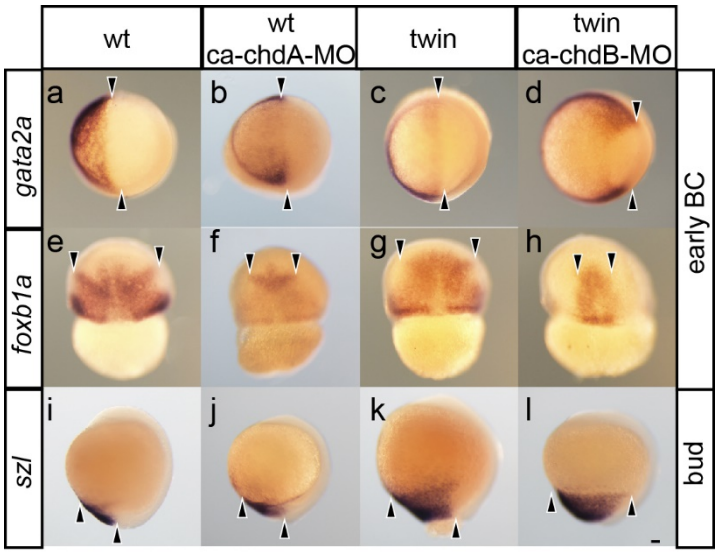



**Supplementary Figure S4: Comparison of chdA mutant and morphant goldfish embryos.**

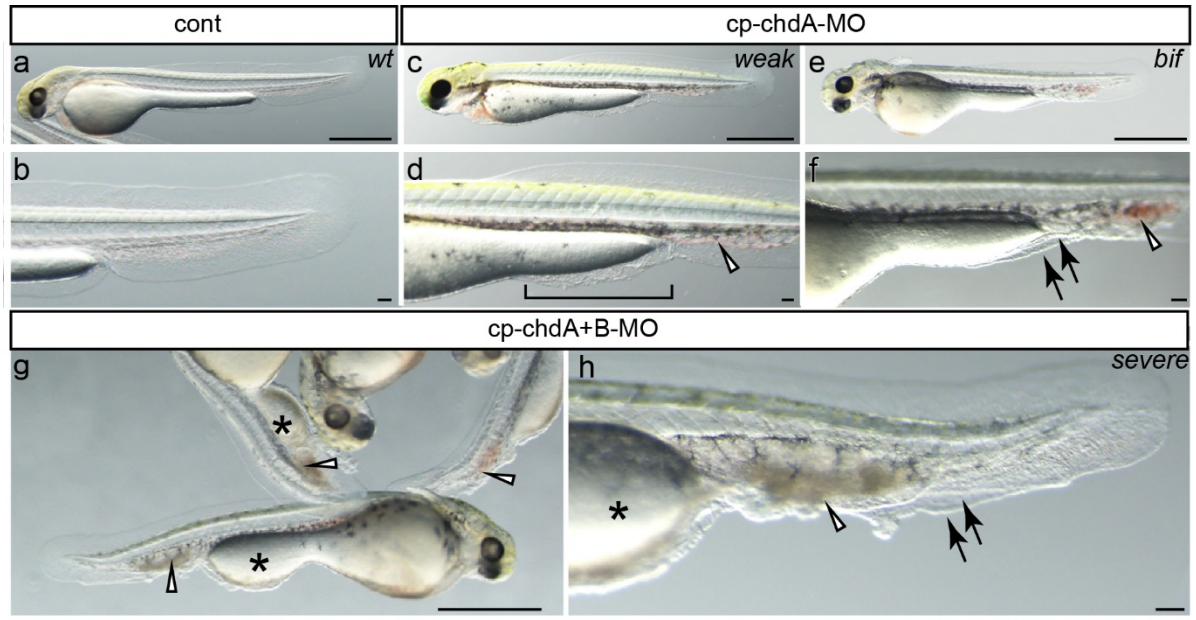
(**a**, **b**) Late pharyngular stage embryo of twin-tail goldfish (*chdA* mutant; *chdA*$^{E127X/E127X}$). (**c**, **d**) Late pharyngular stage embryo of *chdA* morphant goldfish. Panels **b** and **d** are magnified views of panels **a** and **c**, respectively. White arrowheads and black arrows indicate enlarged blood islands and bifurcated fin folds, respectively. Scale bars = 1mm (**a**, **c**), 0.1mm (**b**, **d**).



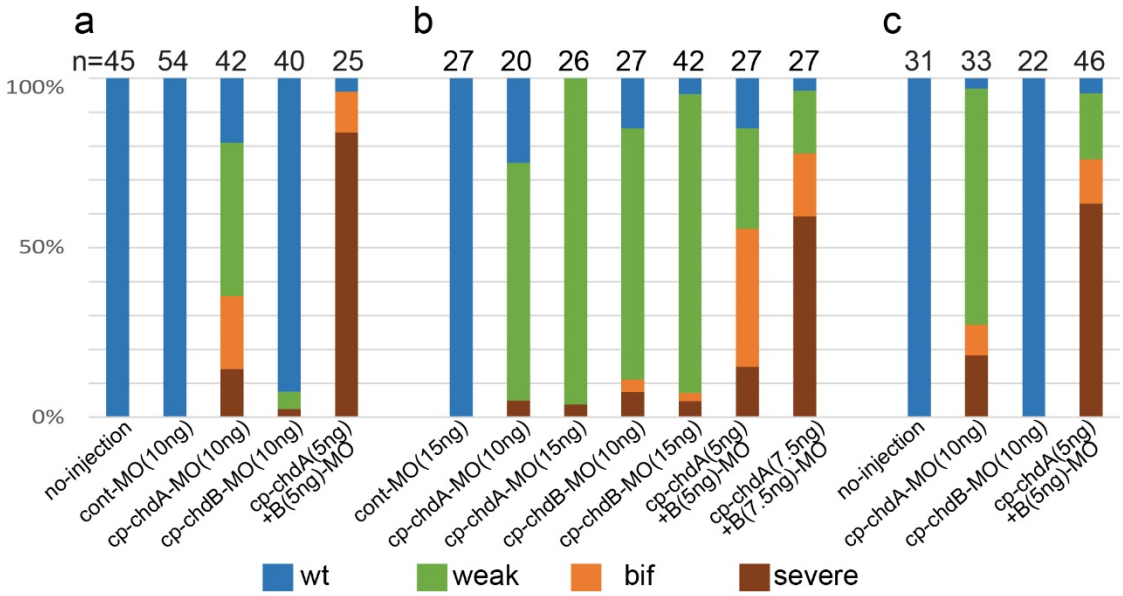

**Supplementary Figure S5: Phenotype analyses following the co-injection of *chdA* MO and *chdB* mRNA into wildtype goldfish embryos.** Co-injection of *chdA* MO and *chdB* mRNA (**a** and **b**). The *chdA* morpholino knock down phenotype was rescued by *chdB* mRNA in a dose-dependent manner (**a** and **b**), suggesting that *chdA* MO depleted *chdA* function in goldfish during early embryogenesis. Dose-dependent effect of *chdA* MO injection (**c** and **d**). The effects of injecting 5 ng or 10 ng *chdA* MO per embryo were similar and reproducible in both clutches (**c** and **d**), suggesting that MO concentrations of 5 ng per embryo are sufficient to induce the twin-tail phenotype. Embryos of each experiment (a-d) were derived from different sets of parents. Phenotypes of embryos were categorized as follows: i) wildtype (wt); ii) weakly-ventralized (weak), and iii) bifurcated fin fold (bif). These criteria were based on previous phenotypic descriptions[9].



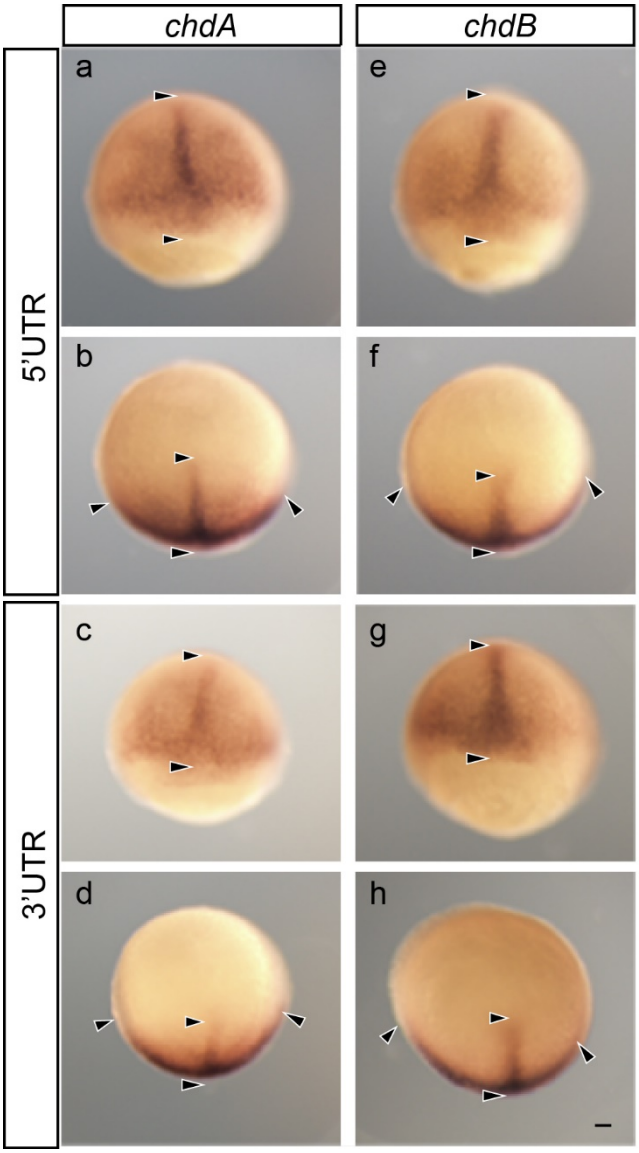

**Supplementary Figure S6: Comparison of gene expression patterns of *chordin* gene deficient goldfish.** Expression patterns of *gata2a* (animal pole view, **a**-**d**), *foxb1a* (dorsal view, **e**-**h**), and *szl* (lateral view, **i**-**l**). Expression patterns of *gata2a*, *szl*, and *fox1ba* in wildtype goldfish embryos (**a**, **e**, **i**), *chdA* morphant goldfish (**b**, **f**, **j**), twin-tail goldfish (**c**, **g**, **k**), and *chdB* morpholino-injected twin-tail goldfish embryos (**d**, **h**, **l**) are shown. Areas of gene expression are indicated by black arrowheads. All panels are shown at the same magnification. Scale bars = 0.5mm.




**Supplementary Figure S7: Common carp morphant phenotypes.** (**a-h**) Morphology of late stage embryos of control (**a**, **b**), *chdA* morphant (**c-f**), and *chdA* and *–B* double morphants (**g**, **h**). Panels **b**, **d**, **f**, and **h** are magnified views of panels **a**, **c**, **e**, and **g** respectively. Black arrows, white arrowheads, asterisks, and brackets in panels **d**, **f**, **g**, and **h** indicate bifurcated fin folds, enlarged blood islands, expanded yolk extensions, and malformed fin fold, respectively. Panels **e**, **f**, **g**, and **h** are identical to panels **a**, **b**, **c**, and **d** in **Fig. 4**, respectively. Scale bars=1mm (**a, c, e, g)**, 0.1mm (**b, d, f, h**).




**Supplementary Figure S8: Proportions of observed phenotypes in *chordin* gene morphant common carp late embryos.** Data from three different batches are shown (**a**-**c**). Higher doses of MO reagent(s) (15 ng/embryo) were injected in the second batch of experiments (**b**). Injected morpholino reagent(s) and dose are indicated at the bottom of the graphs. The total number of phenotyped *chdA*, *-B*, and *A+B* double morphants in the first to third experiments (**a**-**c**) were 121, 131, and 125, respectively.




**Supplementary Figure S9: Comparison of *chdA* and *–B* gene expression patterns in common carp.** Expression patterns of *chdA* (**a**-**d**) and *–B* (**e**-**h**) genes were examined from dorsal (**a**, **e**, **c**, **g**) and animal pole (**b**, **f**, **d**, **h**) views in early blastopore closure stage embryos, using probes designed against different cDNA sequence regions; 5'UTR (**a**, **b**, **e**, **f**) and 3'UTR (**c**, **d**, **g**, **h**). Black arrowheads indicate gene expression areas. Scale bar = 0.1mm. All panels are shown at the same magnification.

## Supplementary Table S1: Primer list

| species | gene | primer names | sequence (5' -> 3') |
|---|---|---|---|
| goldfish and common carp | *foxb1a* | foxb1a-f1 | CCYAGACCCGGGAGAAACAC |
| goldfish and common carp | *foxb1a* | foxb1a-f2 | AGAAACCGCCGTACTCCTACA |
| goldfish and common carp | *foxb1a* | foxb1a-r1 | CTCTTGCCTTGATTGTATAGCCA |
| goldfish and common carp | *foxb1a* | foxb1a-r2 | TGTCAGTGCACCGCGACC |
| goldfish and common carp | *gata2a* | sp6p-gata2a-f1 | CAGTGAATTGATTTAGGTGACACTATAGAAGTGATGGAGGTCGCGGCCGATCAGTCT |
| goldfish and common carp | *gata2a* | t7p-gata2a-r1 | CAGTGAATTGTAATACGACTCACTATAGGGAGAGTGAGGCTCGGCCCAGGCCGGA |
| common carp | *chdA* | cc-chdA-5rr1 | GTGCGACGCCGCTGGAGCTCGG |
| common carp | *chdA* | cc-chdA-5rr2 | CGTGATGGAGAAGGTCAGGCTCGTTCGAGCAA |
| common carp | *chdA* | cc-chdA-gf2 | AAACAGGACTGTCCCGATCCGAC |
| common carp | *chdB* | cc-chdB-5rr1 | CCCGAGCGACGCCGCTGGAGTCCATT |
| common carp | *chdB* | cc-chdB-5rr3 | ATCGGAGTCCAGGAAGGTGAGGA |
| common carp | *chdB* | cc-chdB-5rr4 | GAGCGACGCCGCTGGAGTCCATTAC |
| common carp | *chdB* | cc-chdB-gf2 | AAACAGGATTGTCCCGATCCGTC |
| common carp | *chdA and -B* | cc-chd-sf1 | GCAACTCGGGGAGAAAGGAGG |
| common carp | *chdA and -B* | cc-chd-sr1 | TGTTCTCCTCAGAGCTGATGTAGG |
| common carp | *chdA* | Carpio-chdA-SP6-F3 | CAGTGAATTGATTTAGGTGACACTATAGAAGTGGGTGGGAGAAATGAAGTG |
| common carp | *chdA* | Carpio-chdA-T7R | TAATACGACTCACTATAGGGAGACAGACTGACAACATTAGGTGC |
| common carp | *chdB* | Carp-chdB-SP6-F2 | CAGTGAATTGATTTAGGTGACACTATAGAAGTGGCACCTGGCACAACATGGCATCC |
| common carp | *chdB* | Carp-chdB-T7-R1 | TAATACGACTCACTATAGGGAGAGGATTTGAGTGTAAATATTAAGGATTGTAATGAC |
| common carp | *chdA* | Carp-chdA-SP6-5'race-insitu | CAGTGAATTGATTTAGGTGACACTATAGAAGTGATCAGTGCGCACTGAC |
| common carp | *chdA* | Carp-chdA-T7-5' race-insitu | TAATACGACTCACTATAGGGAGAGATGTAGGATCTGTCATTG |
| common carp | *chdB* | Carp-chdB-SP6-5'race-insitu | CAGTGAATTGATTTAGGTGACACTATAGAAGTGCTGTATAAGACCCCGT |
| common carp | *chdB* | Carp-chdB-T7-5' race-insitu | TAATACGACTCACTATAGGGAGAGTGAATCTGGCCCGAGCG |
| common carp | *szl* | Carp-sizzled-SP6-F2 | CAGTGAATTGATTTAGGTGACACTATAGAAGTGGTCAGGATCATGCATCTGTCT |
| common carp | *szl* | Carp-sizzled-T7-R880 | TAATACGACTCACTATAGGGAGAGAGCCACGGGAACAGACTGG |
| common carp | *chdA* | Carp-chdA-full length-Fw d1 | CACATTCACTGAGCTATATCCAGCAGAACCTCGC |
| common carp | *chdA* | Carp-chdA-full length-Rev1 | TTTTAAAACTTTGTTTATTCATCATAATTAATAAATATGC |
| common carp | *chdB* | Carp-chdB-full length-Fw d2 | CCCATCCAGCCAGAGACAGAACCCTTGATA |
| common carp | *chdB* | Carp-chdB-full length-Rev2 | CATGTGATTGTTACAGGTGTGATGATGTATG |