Supporting Information

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SI Methods

Phylogeny of Cichlid Irx1. Annotated sequences of zebrafish Irx proteins (ENSDARP00000007800, ENSDARP00000073569, ENSDARP00000016693, ENSDARP00000069381, ENSDARP00000042270, ENSDARP00000051692, ENSDARP00000052337, ENSDARP00000043856, ENSDARP00000045627, ENSDARP00000025947, NP_001001405.1), as well as the Irx1 proteins of *Fugu* (ENSTRUP00000018488, ENSTRUP00000029525), *Tetraodon* (ENSTNIP00000012127), edaka (ENSORLP00000006499, ENSORLP00000017930), stickleback (ENSGACP00000008692, ENSGACP00000011891), and an outgroup from *C. elegans* (C36F7.1), were downloaded from the Refseq (release 31) and Ensembl (version 50) databases (1, 2). Together with the cichlid Irx sequence, a multiple sequence alignment was generated using Clustalw version 2.0.10 (3). Phylogenetic relationships between the gene sequences were determined using various programs of the PHYLIP package (version 3.67) (4). Here 1,000 bootstrap replicates of the multiple sequence alignment were generated using Seqboot, pairwise distance measurements were calculated using Protdist, neighbor-joining trees were constructed using Neighbor, and the final consensus tree, together with the bootstrap percentages, was generated with Consense.

1. Pruitt KD, Tatusova T, Maglott DR (2007) NCBI reference sequences (RefSeq): A curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* 35 (Database issue):D61–D65.
2. Flicek P, et al. (2008) Ensembl 2008. *Nucleic Acids Res* 36 (Database issue):D707–D714.
3. Larkin MA, et al. (2007) Clustal W and clustal X version 2.0. *Bioinformatics* 23: 2947–2948.
4. Felsenstein J (2007) *PHYLIP (Phylogeny Inference Package), version 3.67* (University of Washington, WA).

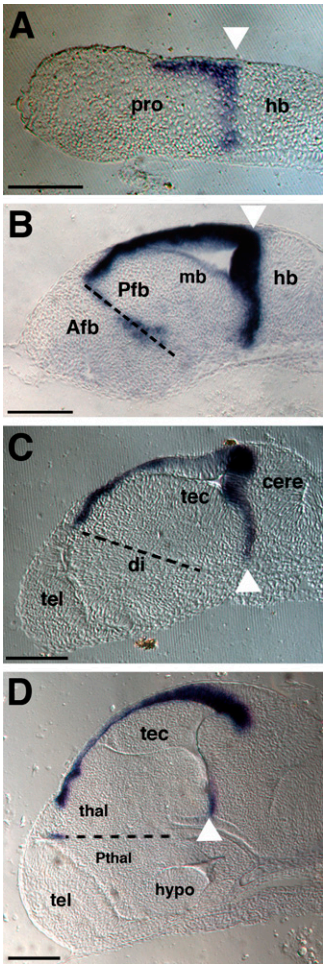

Fig. S1. Staging the cichlid brain. (*A*) ISH showing *wnt1* gene expression for *M. conophorus* (MC), at late stage 9. (*B–D*) The same species, gene expression, and layout as *A*. *B*, stage 12; *C*, stage 14; *D*, stage 16. White arrowheads mark the position of the MHB; dashed lines mark the position of the ZLI. (Scale bar: 100 μm.) hb, hindbrain; pro, prosencephalon; mb, midbrain; Afb, anterior forebrain; Pfb, posterior forebrain; cere, cerebellum; tec, tectum; di, diencephalon; tel, telencephalon; thal, thalamus; Pthal, prethalamus; hypo, hypothalamus. All panels show a parasagittal section with the greatest dorsoventral extent, with anterior to the left.

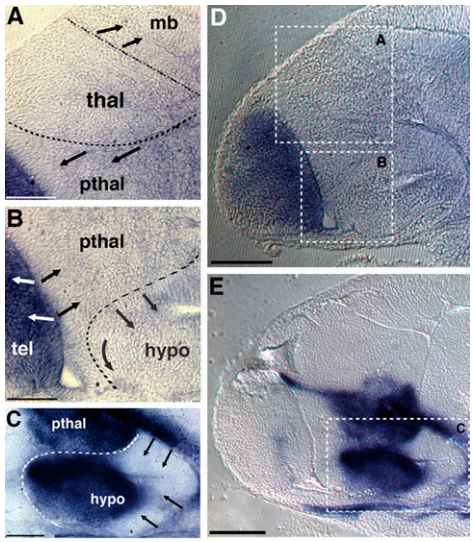

Fig. S2. Measuring the embryonic forebrain at stage 16. Panels *A*–*C* are close-up views of boxes A–C in *D* and *E*. (*A*) Cellular morphology and behavior (marked by arrows) that differentiate the thalamus (thal) from the midbrain (mb) and rest of the forebrain. (*B* and *C*) The other three compartments measured in the forebrain, defined both by gene expression (tel in *B* and pthal and hypo in *C*) and cellular behavior (arrows). The dashed lines in *B* and *C* mark the difference between the prethalamus (pthal) and hypothalamus (hypo). (*D*) ISH of the gene *foxg1* in the nonmbuna, *C. borleyi*. *foxg1* differentiates the telencephalon (tel) from the pthal. (*E*) ISH of the gene *shh* (*M. zebra*), which marks the hypo, as well as the ZLI, the boundary between the pthal and thal. (Scale bars: 50 μm in *A*–*C*; 100 μm in *D* and *E*). All panels are parasagittal sections, with anterior to the left.

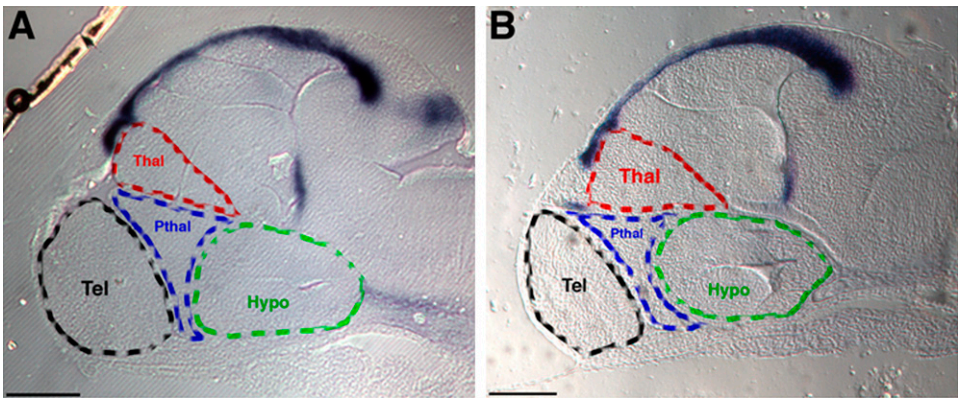

Fig. S3. Stage 16 brains differ between mbuna and nonmbuna. (*A*) ISH of the gene *wnt1* in the mbuna, *M. zebra* (MZ). The red (thalamus), blue (prethalamus), black (telencephalon) and green (hypothalamus) dashes outline the four compartments of the forebrain measured. (*B*) The same gene expression and dashed outlines as *A*, but in the nonmbuna, *M. conophorus* (MC). The brains of mbuna (*A*) exhibit larger telencephala and smaller thalami than their nonmbuna counterparts (*B*) (Table 1). (Scale bar: 100 μm.) Panels are parasagittal sections, with anterior to the left.

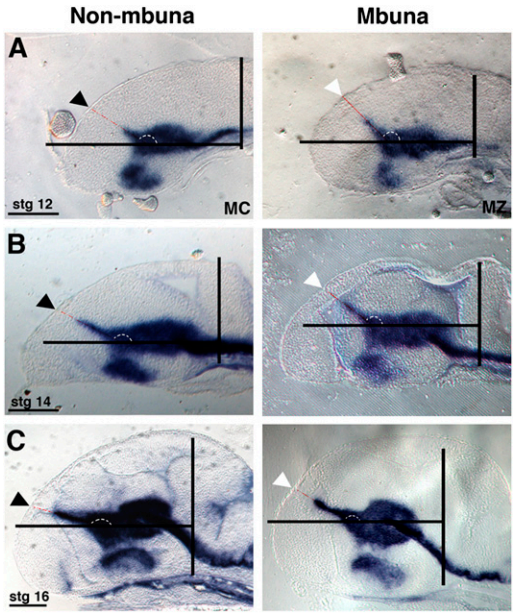

Fig. S4. The difference in angle of the ZLI, between lineages mbuna versus nonmbuna, is maintained throughout ontogeny. ISH for the gene *shh* depicting the angle of the ZLI across three developmental stages: 12 (*A*), 14 (*B*), and 16 (*C*); see Fig. 3. The ZLI is marked by the black arrowhead in nonmbuna (MC) and the white arrowhead in mbuna (MZ). The dotted red and white lines show the ZLI angle (*Methods*). As development progresses, the initial ZLI wedge narrows into a line and the ZLI angle increases. However, at each stage the average ZLI angle in nonmbuna is greater than that of mbuna (Fig. 3). (Scale bar: 100 μm.) Panels are parasagittal sections, with anterior to the left.

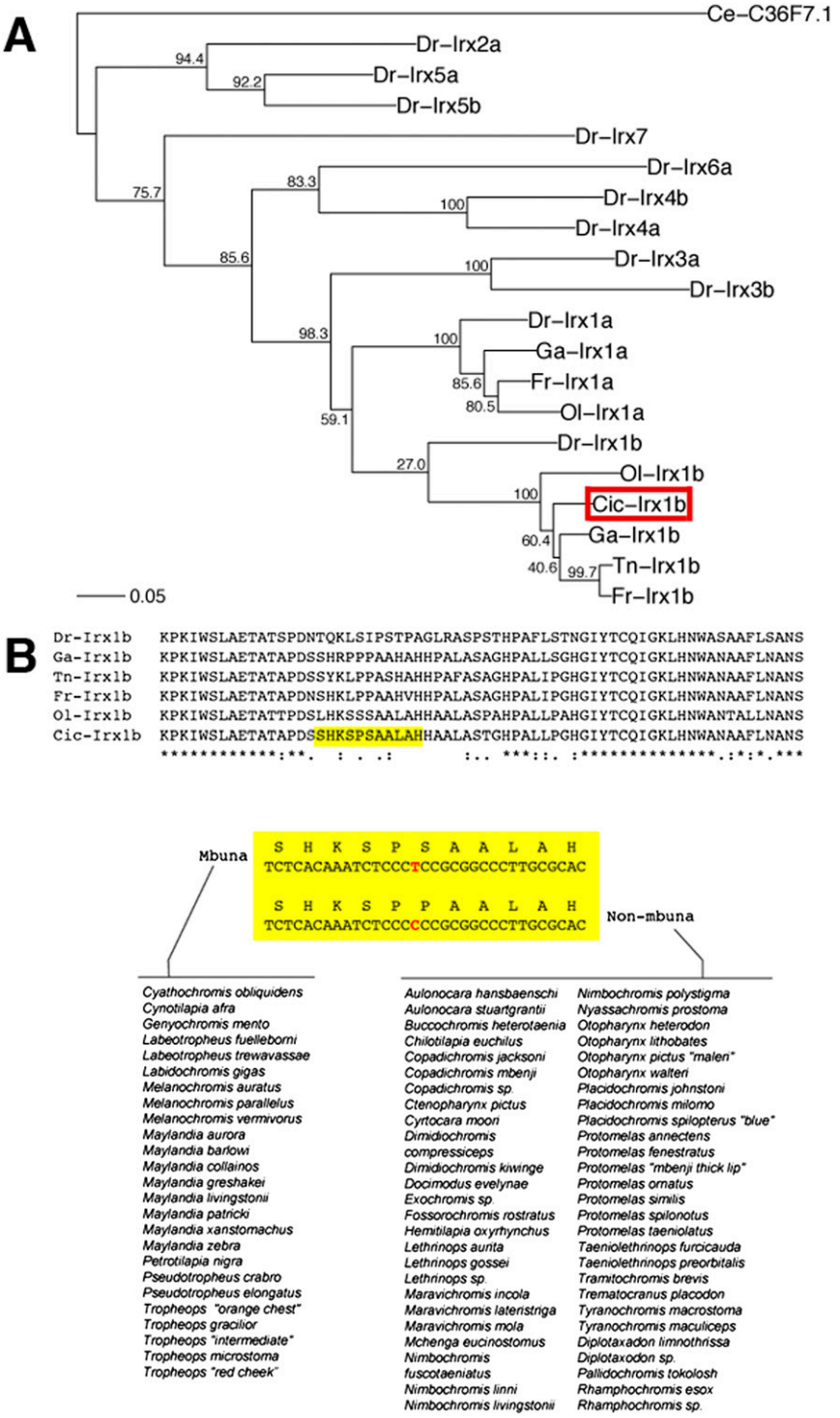

Fig. S5. A SNP in *irx1b* is alternately fixed in mbuna versus nonmbuna cichlids. (*A*) Phylogram of fish Irx sequences shows that cichlid Irx1 is Irx1b. The scale bar represents genetic distance. Numbers at nodes are percentages of 1,000 bootstrap resamplings. Ce, *Caenorhabditis elegans*; Dr, *Danio rerio*; Ga, *Gasterosteus aculeatus*; Fr, *Fugu rubripes*; Ol, *Oryzias latipes*; Cic, cichlid; Tn, *Tetraodon nigroviridis*. (*B*) Local alignment of fish Irx1b amino acid sequences shows the position of the cichlid replacement SNP (yellow; nucleotide is in red) alternately fixed between 25 mbuna species and 52 nonmbuna species.