

## Supplementary Materials

**Table S1. Accession numbers (no.) of *Alx4* coding sequences used in this study.**

| Latin name                        | Common name                     | Accession no.  |
|-----------------------------------|---------------------------------|----------------|
| <i>Alligator mississippiensis</i> | American alligator              | XM_059722770.1 |
| <i>Anas platyrhynchos</i>         | Mallard                         | XM_027458799.3 |
| <i>Apteryx rowi</i>               | Okarito brown kiwi              | XM_026091857.1 |
| <i>Athene noctua</i>              | Little owl                      | XM_074909228.1 |
| <i>Balearica regulorum</i>        | East African grey crowned-crane | XM_075754688.1 |
| <i>Bos taurus</i>                 | Cattle                          | NM_001030304.1 |
| <i>Callithrix jacchus</i>         | Marmoset                        | XM_035262403.2 |
| <i>Caloenas nicobarica</i>        | Nicobar pigeon                  | XM_065636083.1 |
| <i>Calonectris borealis</i>       | Shearwater                      | XM_075152354.1 |
| <i>Camelus bactrianus</i>         | Bactrian camel                  | XM_010964646.3 |
| <i>Canis lupus familiaris</i>     | Dog                             | XM_038424583.1 |
| <i>Caretta caretta</i>            | Loggerhead turtle               | XM_048855228.2 |
| <i>Chelonia mydas</i>             | Green sea turtle                | XM_037900095.2 |
| <i>Chroicocephalus ridibundus</i> | Black-headed Gull               | XM_063334286.1 |
| <i>Colius striatus</i>            | Speckled mousebird              | XM_061998543.1 |
| <i>Crocodylus porosus</i>         | Saltwater crocodile             | XM_019551979.1 |
| <i>Cuculus canorus</i>            | Cuckoo                          | XM_009569981.2 |
| <i>Dermochelys coriacea</i>       | Leatherback sea turtle          | XM_038406932.2 |

|                                 |                           |                |
|---------------------------------|---------------------------|----------------|
| <i>Desmodus rotundus</i>        | Vampire bat               | XM_053925563.1 |
| <i>Dromaius novaehollandiae</i> | Emu                       | XM_026093266.2 |
| <i>Equus asinus</i>             | Donkey                    | XM_044750281.2 |
| <i>Falco peregrinus</i>         | Peregrine falcon          | XM_005241888.3 |
| <i>Felis catus</i>              | Cat                       | XM_003993190.5 |
| <i>Gallus gallus</i>            | Chicken                   | NM_204162.2    |
| <i>Gavia stellata</i>           | Red-throated loon         | XM_059819878.1 |
| <i>Gymnogyps californianus</i>  | California condor         | XM_050898480.1 |
| <i>Hipposideros armiger</i>     | Great roundleaf bat       | XM_019659947.1 |
| <i>Homo sapiens</i>             | Human                     | NM_021926.4    |
| <i>Macaca mulatta</i>           | Rhesus monkey             | XM_001113643.4 |
| <i>Melopsittacus undulatus</i>  | Budgerigar                | XM_034062176.1 |
| <i>Mesoplodon densirostris</i>  | Blainville's beaked whale | XM_060105122.1 |
| <i>Microcebus murinus</i>       | Gray mouse lemur          | XM_012790407.3 |
| <i>Mus musculus</i>             | House mouse               | NM_007442.3    |
| <i>Nothoprocta perdicaria</i>   | Chilean tinamou           | XM_026035139.1 |
| <i>Oryctolagus cuniculus</i>    | Rabbit                    | XM_002709067.5 |
| <i>Otolemur garnettii</i>       | Small-eared galago        | XM_003781249.3 |
| <i>Papio anubis</i>             | Olive baboon              | XM_003909992.5 |
| <i>Passer montanus</i>          | Tree sparrow              | XM_039731037.1 |
| <i>Pelecanus crispus</i>        | Dalmatian pelican         | XM_075713242.1 |

|                               |                              |                |
|-------------------------------|------------------------------|----------------|
| <i>Pelodiscus sinensis</i>    | Chinese soft-shelled turtle  | XM_075927528.1 |
| <i>Physeter macrocephalus</i> | Sperm whale                  | XM_007126417.2 |
| <i>Pogoniulus pusillus</i>    | Red-fronted tinkerbird       | XM_064152543.1 |
| <i>Pongo pygmaeus</i>         | Bornean orangutan            | XM_054439125.2 |
| <i>Pyxicephalus adspersus</i> | African bullfrog             | XM_072422169.1 |
| <i>Rattus norvegicus</i>      | Norway rat                   | NM_001106553.2 |
| <i>Rhea pennata</i>           | Lesser rhea                  | XM_062577235.1 |
| <i>Rhineura floridana</i>     | Florida worm lizard          | XM_061610826.1 |
| <i>Rhinolophus sinicus</i>    | Chinese rufous horseshoe bat | XM_019746387.2 |
| <i>Sceloporus undulatus</i>   | Fence lizard                 | XM_042459735.1 |
| <i>Struthio camelus</i>       | Ostrich                      | XM_068945567.1 |
| <i>Tiliqua scincoides</i>     | Blue-tongued Skink           | XM_066639850.1 |
| <i>Trachemys scripta</i>      | Red-eared slider turtle      | XM_034769613.1 |

**Table S2. Pairwise identity of *Alx4* protein sequences (%).**

| Comparison       | Protein sequence identity |
|------------------|---------------------------|
| Mouse vs Human   | 92.23                     |
| Chicken vs Human | 75.97                     |
| Chicken vs Mouse | 76.60                     |

**Table S3. Length of individual *Alx4* introns (bp).**

| Species | Intron 1 | Intron 2 | Intron 3 | Intron 4 |
|---------|----------|----------|----------|----------|
| Human   | 33938    | 10164    | 2310     | NA       |
| Mouse   | 25266    | 6630     | 1807     | NA       |
| Chicken | 1081     | 19192    | 3678     | 336      |

NA, not applicable; no corresponding fourth intron was annotated in the reference *Alx4* transcripts analyzed for human and mouse.

**Table S4. Comparison of *Alx4* intronic sequences (%).**

| Comparison       | Intron 1 identity | Intron 2 identity | Intron 3 identity | Overall |
|------------------|-------------------|-------------------|-------------------|---------|
| Mouse vs Human   | 57.39             | 51.30             | 52.12             | 57.30   |
| Chicken vs Human | 46.52             | 45.40             | 45.86             | 46.04   |
| Chicken vs Mouse | 44.71             | 44.79             | 45.01             | 45.59   |

**Table S5. Pairwise identity of *Alx4* untranslated regions (%).**

| Comparison       | 5'UTR identity | 3'UTR identity |
|------------------|----------------|----------------|
| Mouse vs Human   | 89.61          | 59.68          |
| Chicken vs Human | 58.33          | 45.85          |
| Chicken vs Mouse | 45.61          | 44.37          |

**Table S6. List of DEGs identified after overexpression of chicken, mouse or human*****Alx4*.**c/m/h*Alx4* indicates chicken/mouse/human *Alx4*.**Table S7. List of 121 common DEGs identified after overexpression of chicken, mouse or human *Alx4*.**c/m/h*Alx4* indicates chicken/mouse/human *Alx4*.**Figures**

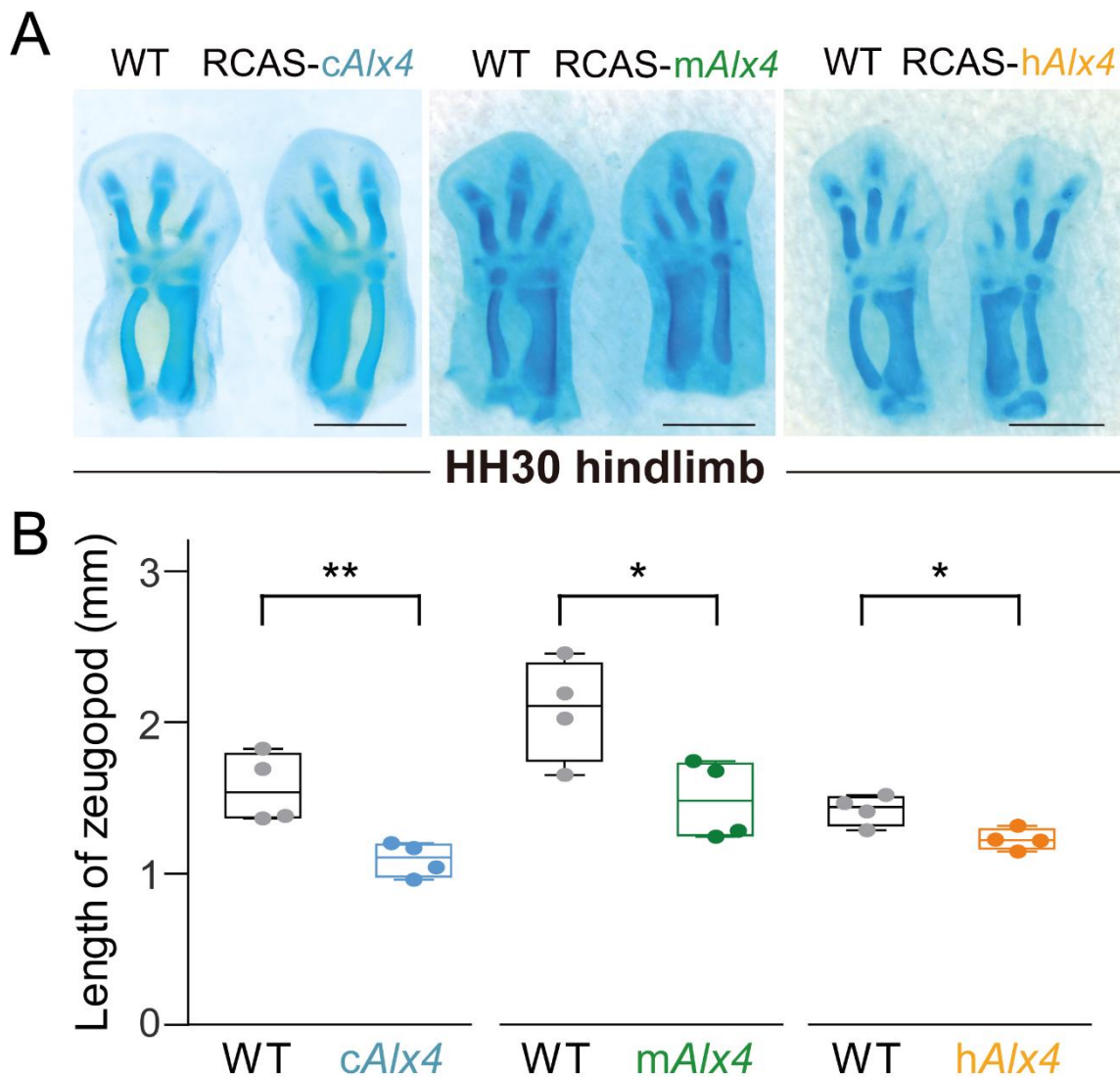


**Figure S1. Cartilage staining and quantification of zeugopod length following overexpression of chicken, mouse, or human *Alx4*.** (A) Hindlimbs were injected with RCAS-*cAlx4*, RCAS-*mAlx4*, or RCAS-*hAlx4* at stage HH17 and collected at stage HH30 for cartilage staining. For each embryo, the uninjected side served as the WT control. Dorsal views are shown with distal up and digit I positioned medially. Bar = 1 mm. (B) Quantification of zeugopod length at HH30. Overexpression of all three *Alx4* orthologs significantly reduced zeugopod length compared with the corresponding WT controls ( $n = 4$  per group), indicating that skeletal shortening is already evident at the cartilaginous stage. Dots represent individual samples. Data are presented as mean  $\pm$  SD, two-tailed Student's *t*-test. \* $P < 0.05$ ; \*\* $P < 0.01$ .

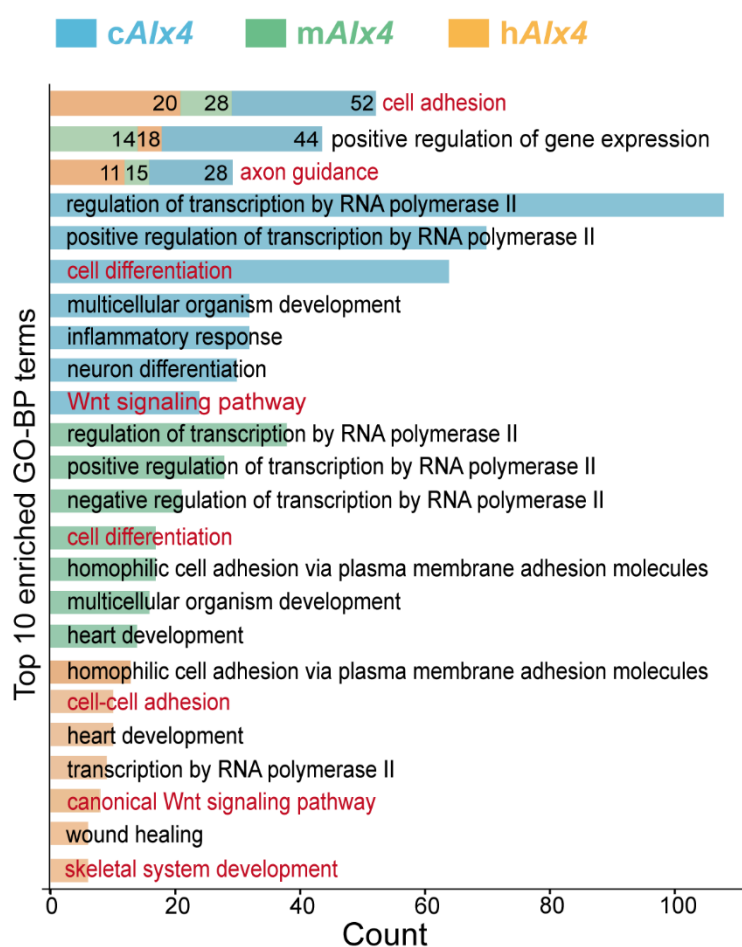


**Figure S2. Results of the GO enrichment analyses for all DEGs.** Terms associated with limb and skeletal development are highlighted in red.