



Figures and figure supplements

Molecular shifts in limb identity underlie development of feathered feet in two domestic avian species

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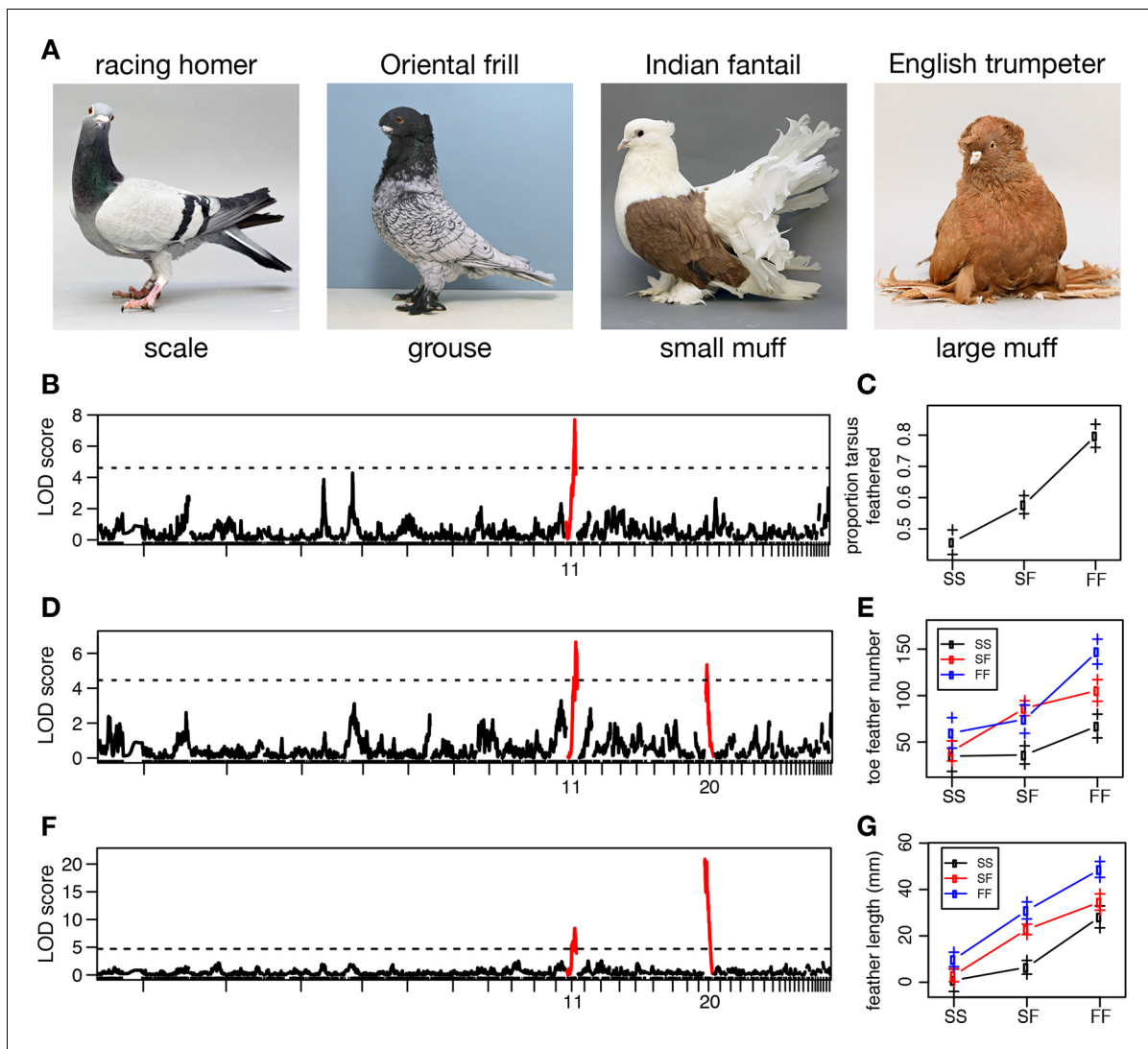


Figure 1. Two QTL differentiate scale- and feather-footed domestic pigeons. (A) Common phenotypes of domestic rock pigeon, in order of increasing foot feathering (left to right): scaled, grouse, small- and large-muffed. (B-F) QTL scans and effect plots: proportion of tarsus feathered (B,C), number of toe feathers (D,E), and length of toe feathers (F,G). Mean phenotypes \pm S.E. are plotted in (C,E,G). For (E) and (G), genotypes at the QTL with the higher LOD score are on the x-axis, and genotypes at the other QTL are inset. See **Tables 1** and **2** for detailed QTL statistics. S, allele from scale-footed grandparent; F, allele from feather-footed grandparent.

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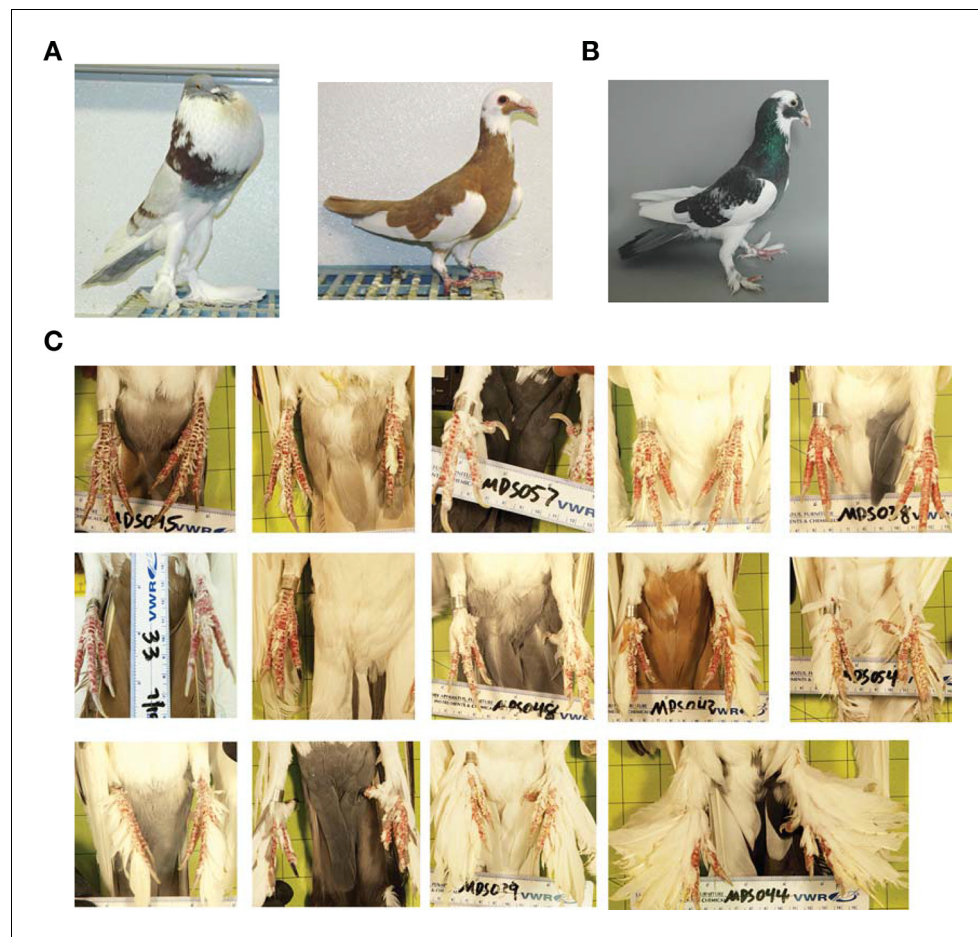


Figure 1—figure supplement 1. Foot-feathering phenotypes of genetic cross. (A) Founder Pomeranian pouter male (muffed) and Scandaroon female (scale-footed). (B) Representative F_1 individual with moderate foot feathering. (C) Representative sample set of F_2 individuals.

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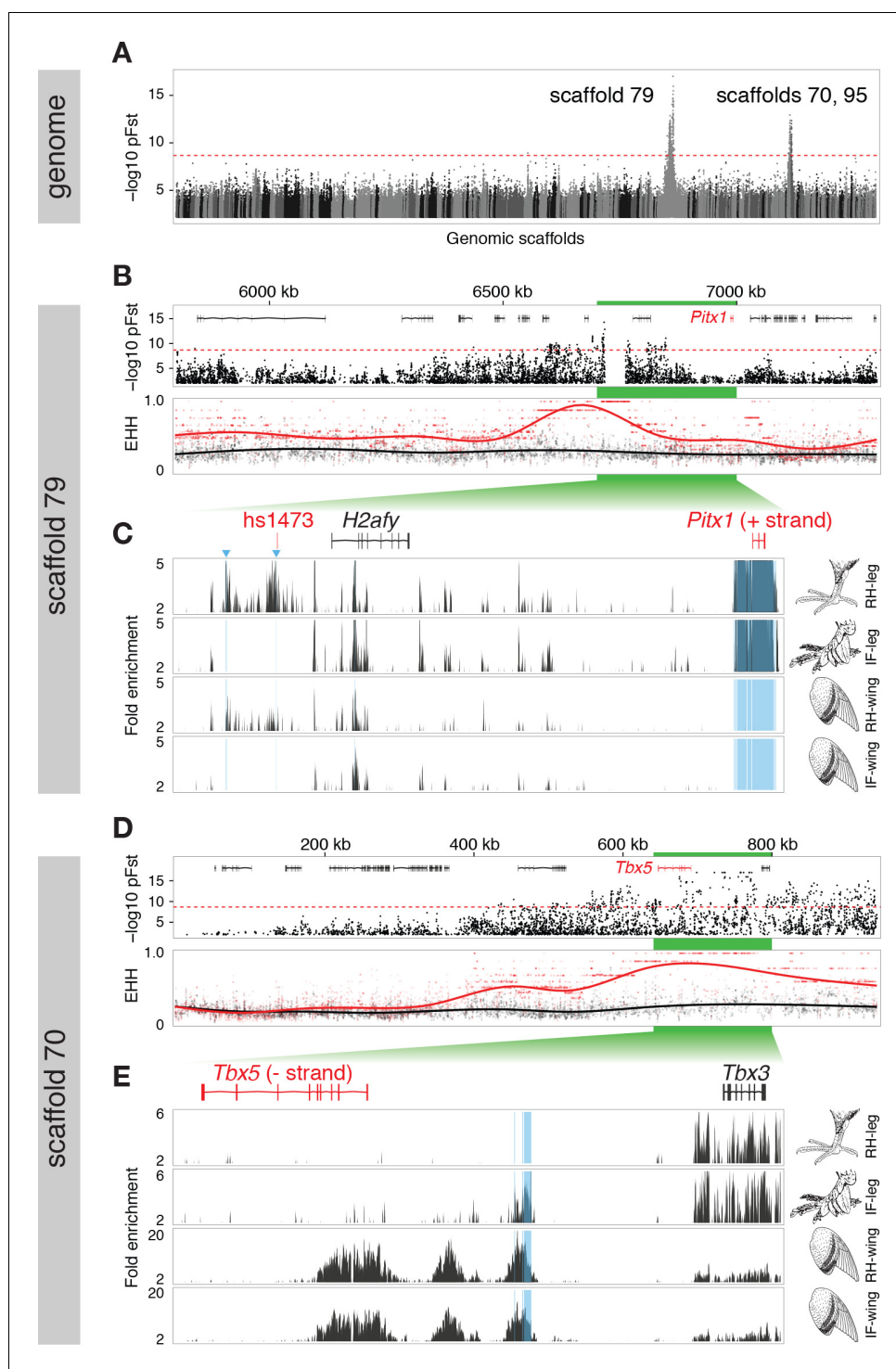


Figure 2. Two regions of genomic differentiation and H3K27ac enrichment distinguish scale- and feather-footed pigeons. (A) Whole-genome pFst comparisons between genomes of feather-footed and scale-footed pigeons. Scaffolds are ordered by genetic position in a linkage map from an F_2 cross (see **Figure 1**). Dashed line, genome-wide significance threshold. (B) pFst and extended haplotype homozygosity (EHH) plots for region of high differentiation on scaffold 79. Feather-footed birds ($n=10$, red in EHH plot) homozygous for a 44-kb deletion are differentiated from scale-footed birds ($n=28$, black) and show extended haplotype homozygosity in this region. Smoothed lines follow a generalized additive model (Wickham, 2009). (C) H3K27ac ChIP-seq enrichment differed significantly between embryonic wing and leg buds of the scale-footed racing homer (RH) in several regions (blue **Figure 2 continued on next page**

Figure 2 continued

shading), including within a 44-kb interval that is deleted in the muffed Indian fantail (IF; blue arrowheads). This deleted region is orthologous to a known human limb enhancer (hs1473). (D) Selection scans show similar patterns of differentiation on scaffold 70 between muffed (n=11, red in EHH plot) and scale-footed birds (n=28, black). (E) H3K27ac ChIP-seq enrichment differed significantly between leg buds of racing homer and Indian fantail embryos in regions immediately 5' of *Tbx5* (blue shading). Foot and wing drawings modified after **Levi (1986)**.

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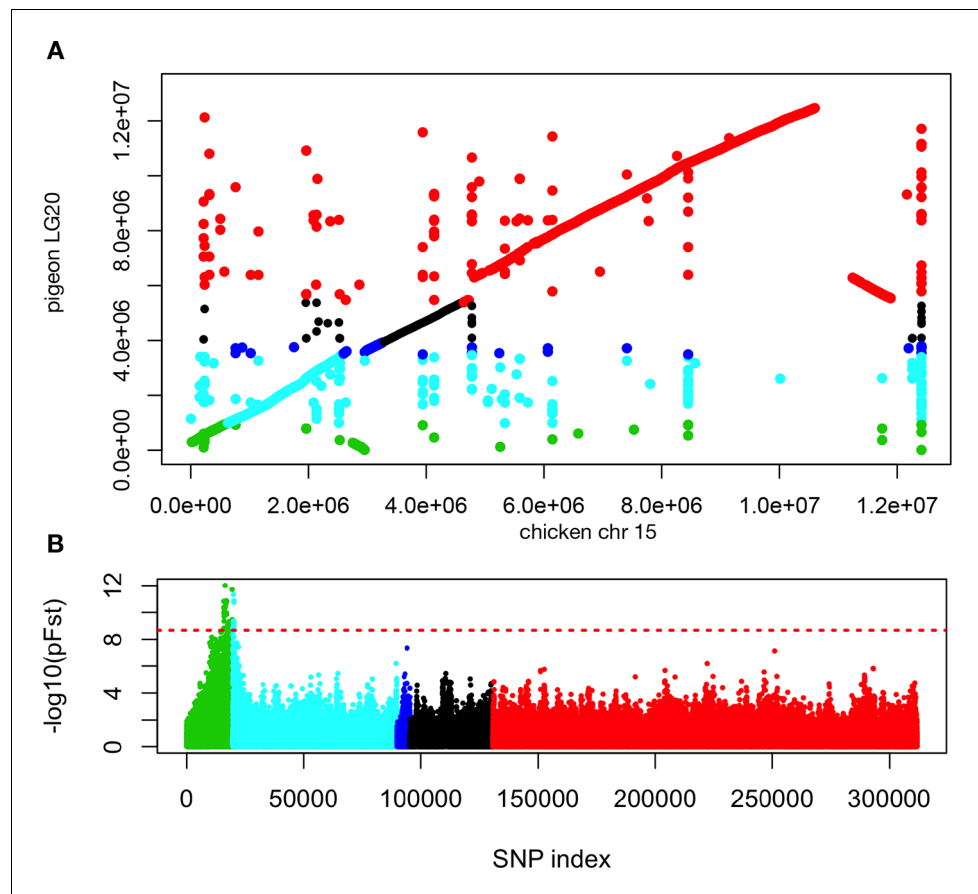


Figure 2—figure supplement 1. Synteny and genomic differentiation of pigeon LG20. (A) Lastz alignment of pigeon scaffolds corresponding to linkage group 20 with chicken chromosome 15. (B) pFst scan of scaffolds from linkage group 20, ordered based on genetic linkage and synteny with chicken chromosome 15. green = scaffold 70, aqua = scaffold 95, blue = scaffold 737, black = scaffold 143, red = scaffold 20. Red line = whole genome significance threshold (2.11×10^{-9}).

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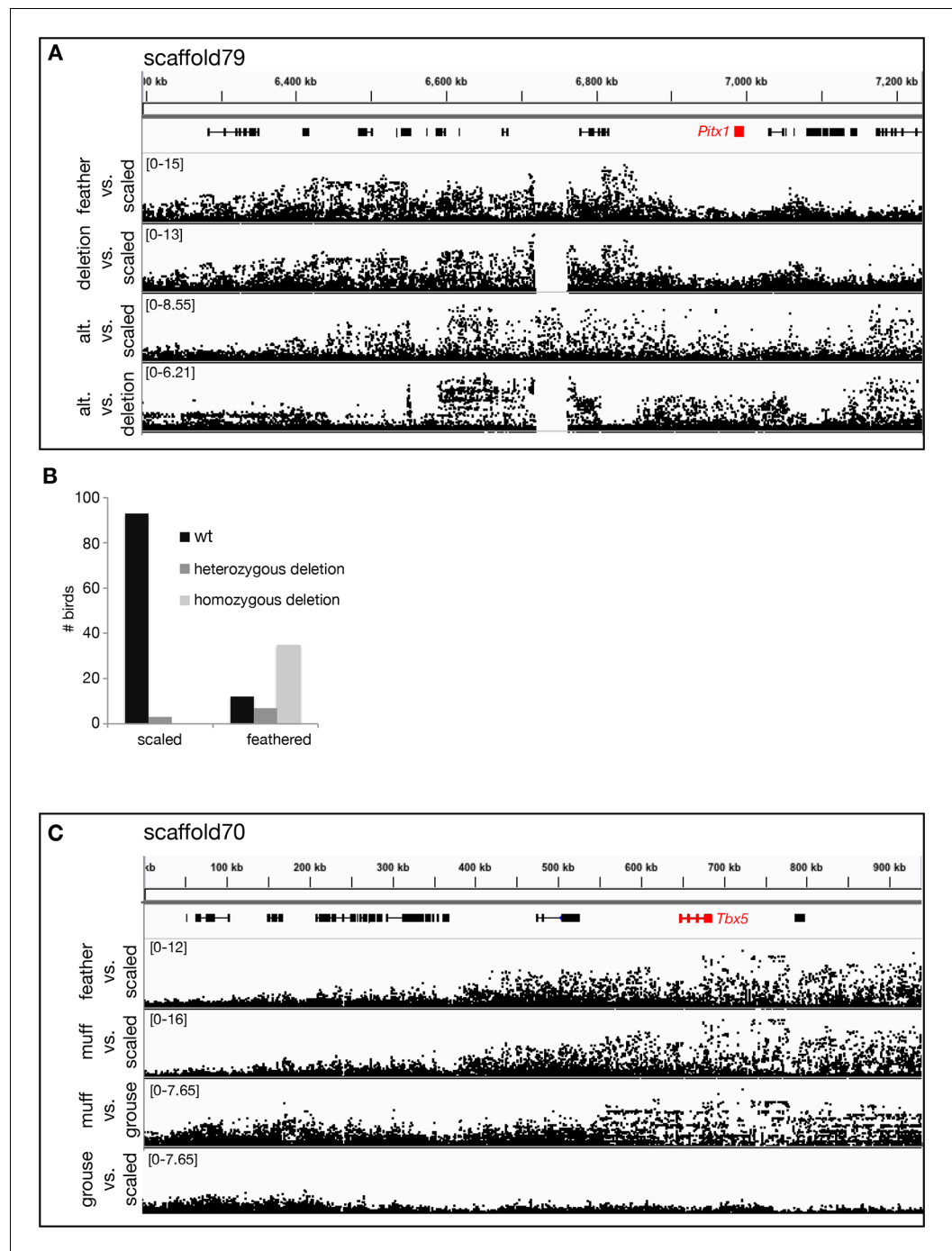


Figure 2—figure supplement 2. Genomic association scans. (A) pFst scans on scaffold 79 showing all feather-footed birds vs. all scale-footed birds, feather-footed birds homozygous for deletion vs. scale-footed birds, feather-footed birds lacking the deletion (alt) vs. scale-footed birds, and feather-footed birds lacking the deletion (alt) vs. feather-footed birds homozygous for the deletion. (B) Histogram of genotypes for scaffold 79 deletion, scale-footed vs. feather-footed phenotype. (C) pFst scans on scaffold 70 showing all feather-footed birds vs. scale-footed birds, muff birds vs. scale-footed birds, muff vs. grouse birds, and grouse vs. scale-footed birds. Range of $-\log_{10}(\text{pFst})$ values on left side of each plot.

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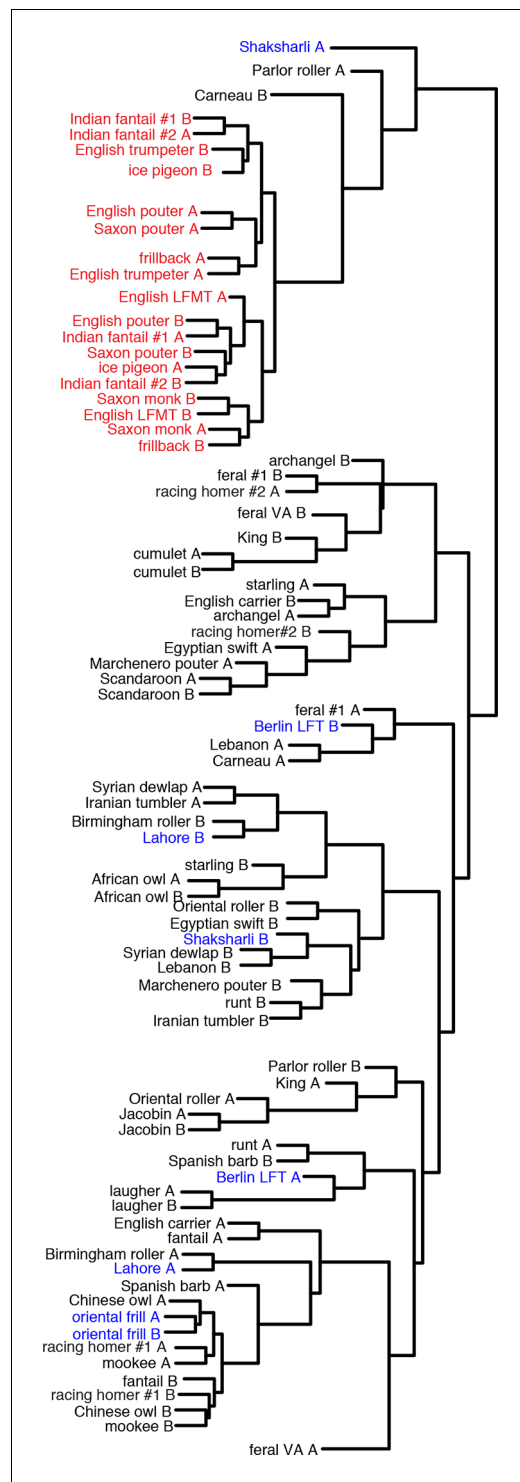


Figure 2—figure supplement 3. Haplotype diagram of scaffold 70 candidate interval. Haplotypes clustered based on binary distance from muff (red), grouse (blue), and scale-footed (black) birds.

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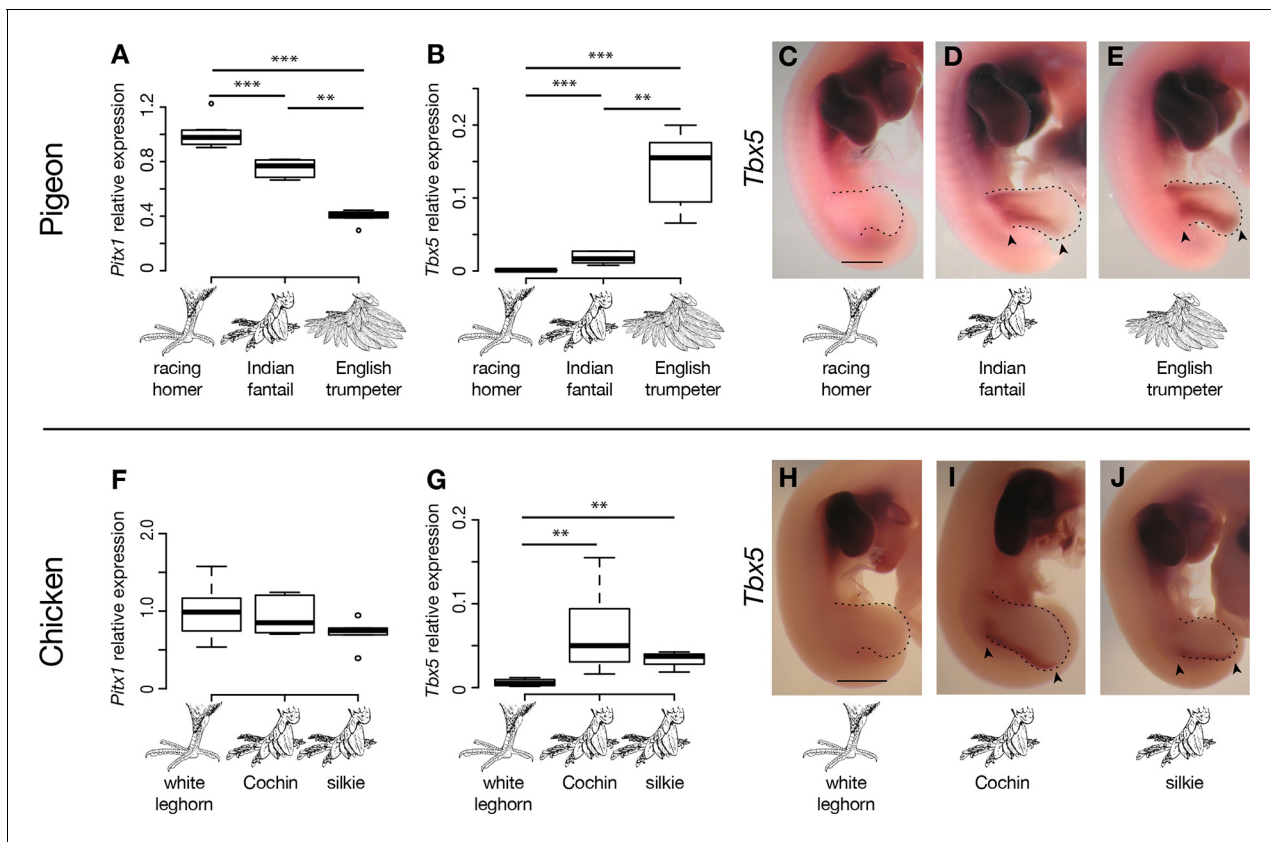


Figure 3. Limb-type gene expression varies among feathered and scaled pigeons and chickens. (A,B,F,G) qRT-PCR analyses of *Pitx1* and *Tbx5* expression in HH25 hindlimbs of pigeon (A,B) and chicken (F,G). Boxes span 1st to 3rd quartiles, bars extend to minimum and maximum observed values if within 1.5 times the interquartile range of the box, circles indicate values outside of this range, black line indicates median. **= $p < 0.01$, ***= $p < 0.001$. (C-E, H-J) RNA in situ hybridization for *Tbx5* expression in HH25 embryos of racing homer (C), Indian fantail (D), and English trumpeter (E) pigeons; and white leghorn (H), Cochin (I), and silk (J) chickens. Arrowheads indicate sites of ectopic *Tbx5* expression. Scale bar = 1 mm.

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The following source data is available for figure 3:

Source data 1. Source data from quantitative RT-PCR experiments.

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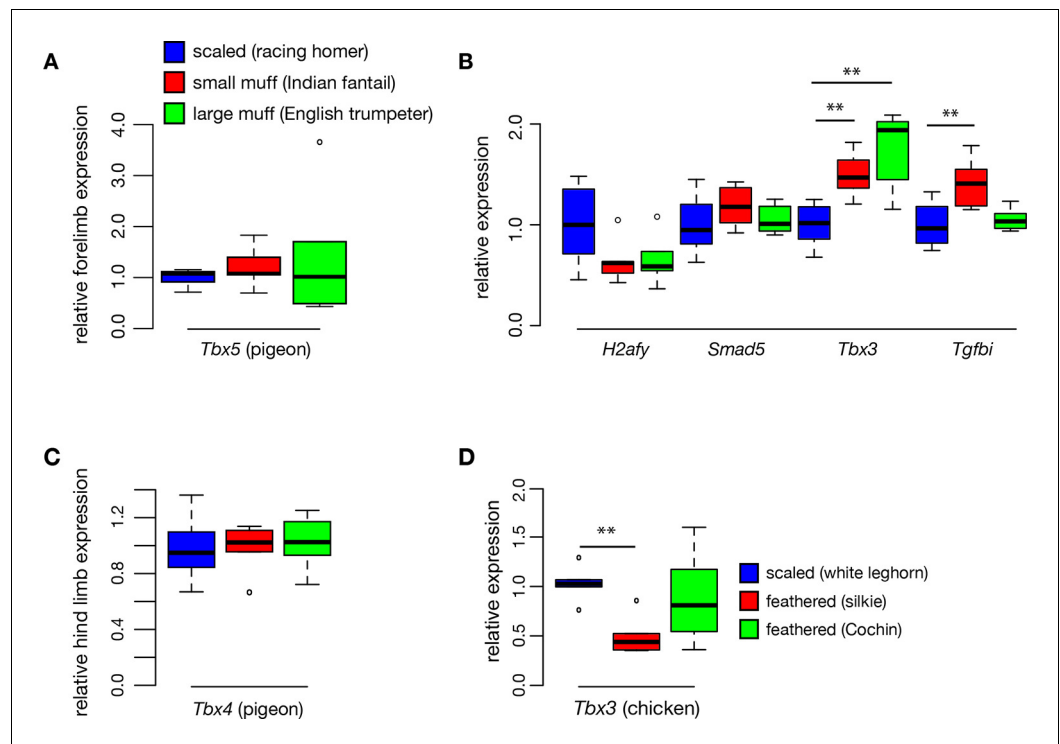


Figure 3—figure supplement 1. Quantitative RT-PCR expression analyses. (A) Expression levels of *Tbx5* are similar among racing homer, Indian fantail, and English trumpeter HH25 forelimb buds ($n = 6$ samples each for all pigeon and chicken comparisons). (B) Expression comparisons for additional genes within candidate intervals on scaffolds 70 and 79. See main text for further discussion of *Tbx3* results. (C) Expression levels of *Tbx4* are similar among racing homer, Indian fantail, and English trumpeter hindlimbs. (D) Expression levels of *Tbx3* are reduced in HH25 hindlimb buds of one (but not both) feather-footed chicken breeds.

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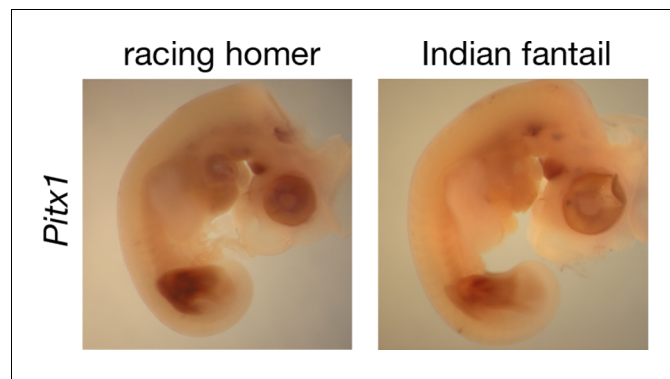


Figure 3—figure supplement 2. Spatial expression pattern of *Pitx1* is similar in hindlimb buds of scaled-foot and feathered-foot embryos. Whole-mount RNA in situ hybridization results for *Pitx1* expression in racing homer (scaled-foot, left) and Indian fantail (feathered-foot, right) HH25 embryos.

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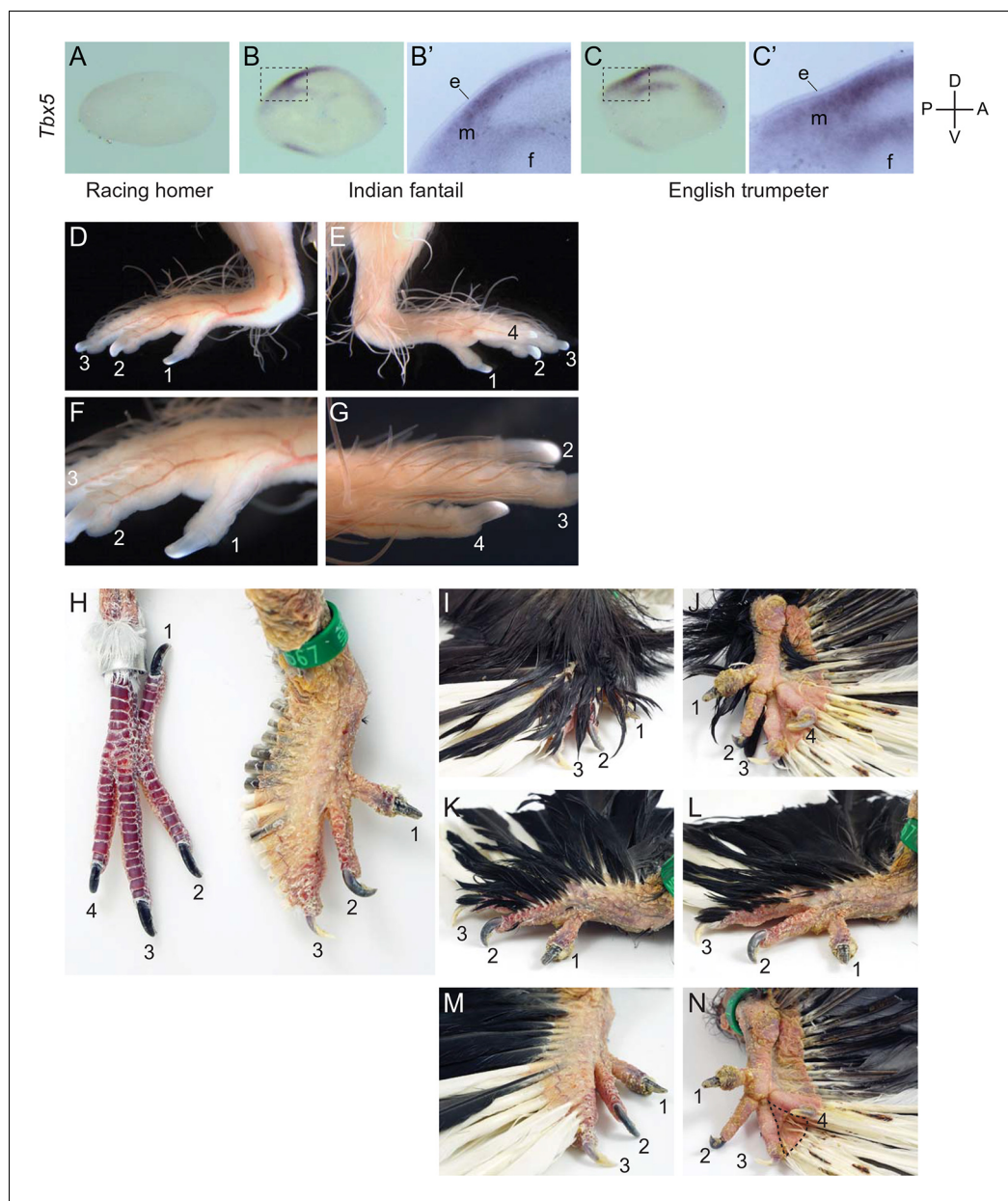


Figure 3—figure supplement 3. Ectopic hindlimb expression of *Tbx5* and epidermal transformations in embryos and adults. (A–C') Vibrotome sections through HH25 pigeon hindlimbs shown in **Figure 3C–E**. *Tbx5* expression is absent from the hindlimb buds of the wild-type racing homer (A), but is present in the mesenchyme (most notably in the posterior-dorsal region) of the muffed Indian fantail and English trumpeter breeds. Magnified images (B', C') show that staining is limited to the mesenchyme (m) and does not extend into the ectoderm (e). Sections were cut in the zeugopod region; magnified sections include the fibula condensation (f). (D–G) Embryonic hindlimb of an English trumpeter at approximately 15 days of incubation. Digits are numbered from medial (1) to lateral (4). Feather primordia are visible as string-like structures throughout the limb in medial (D) and lateral (E) views. Feathers are longer on the lateral digits (G) than on the medial digits, and these differences persist into adulthood. (H) Comparison of adult feet of a racing homer (scaled, wild-type, left) and an English trumpeter (feathered, right). All small feathers have been plucked from the English trumpeter foot, and large feathers have been trimmed down to their insertions in the skin. Note that digit 4 in the English trumpeter is not visible due to the expanded skin on digit 3, and unlike the backward-facing digit 1 of the racing homer, digit 1 faces forward and medially. (I–N) Details of feather size and distribution in the foot of an English trumpeter. Distal ends of long feathers were clipped and therefore full adult feather size is not represented in these images. The toes are barely visible in a dorsal view of an intact foot (I), and the insertion of large feathers on the lateral side of the foot is

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visible in ventral view (J). Small feathers were plucked from digit 1 and the medial metatarsus to reveal the small feathers inserting on digit 2 in medial view (K), and feathers were plucked from digit 2 to reveal small feathers inserting on dorsal digit 3 and metatarsus (L). Removal of all small feathers shows the lateral insertion points of large feathers on digit 3 in dorsal view (M). Substantial expansion of lateral skin accommodates the insertion of these large feathers, and digit 4 is hidden from view. Similar skin expansion and large feather insertions characterize digit 4 and the lateral metatarsus (N, ventral view). Soft tissue webbing joins digits 3 and 4 (dashed line), as described by **Darwin, 1868**.

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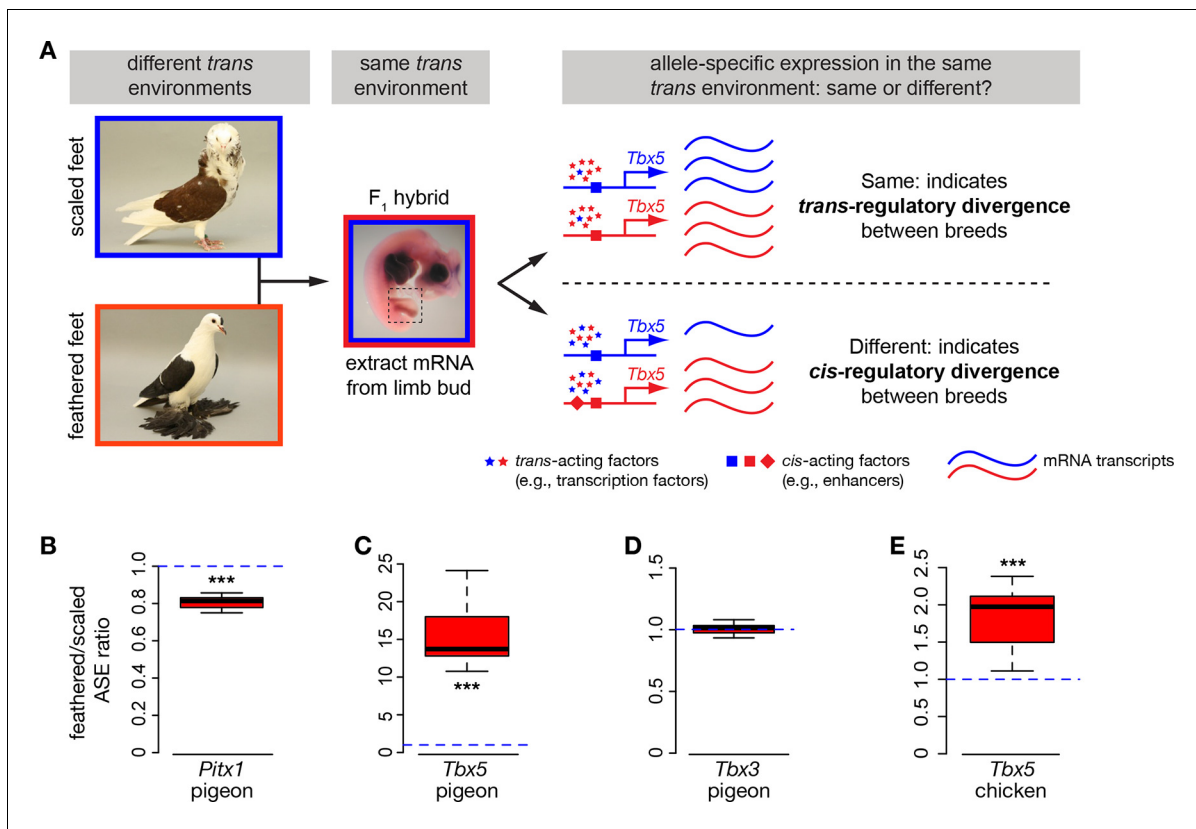


Figure 4. Allele-specific expression (ASE) assays demonstrate cis-regulatory changes in *Pitx1* and *Tbx5*. **(A)** Schematic of ASE assay using *Tbx5* expression as an example. Differences in *Tbx5* expression between scale-footed and feather-footed breeds could be due to *trans*- and/or *cis*-acting mutations. If expression differences between parental breeds are due to *trans* changes only (stars), then expression of the two *Tbx5* alleles in hybrid embryos will be the same (top right). In contrast, if *cis*-regulatory changes underlie differences in *Tbx5* expression between the parental breeds, then expression of the two *Tbx5* alleles in hybrid embryos will be different (bottom right). **(B-D)** ASE assay in hybrid hindlimb buds indicate *cis*-regulatory divergence between scale-footed (Old Dutch Capuchine) and muffed (fairy swallow) pigeon breeds in *Pitx1* **(B)** and *Tbx5* **(C)**, but not in *Tbx3* **(D)**. Dashed blue line indicates null hypothesis of equal expression of alleles. **(E)** ASE assay in hybrid hindlimb buds indicate *cis*-regulatory divergence in *Tbx5* between feather-footed (silkie) and scale-footed (white leghorn) chicken breeds. Boxes in **(B-E)** span 1st to 3rd quartiles, bars extend to minimum and maximum observed values if within 1.5 times the interquartile range of the box, circles indicate values outside of this range, black line indicates median. **p < 0.01 ***p < 0.005.

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The following source data is available for figure 4:

Source data 1. Source data from pyrosequencing ASE experiments.

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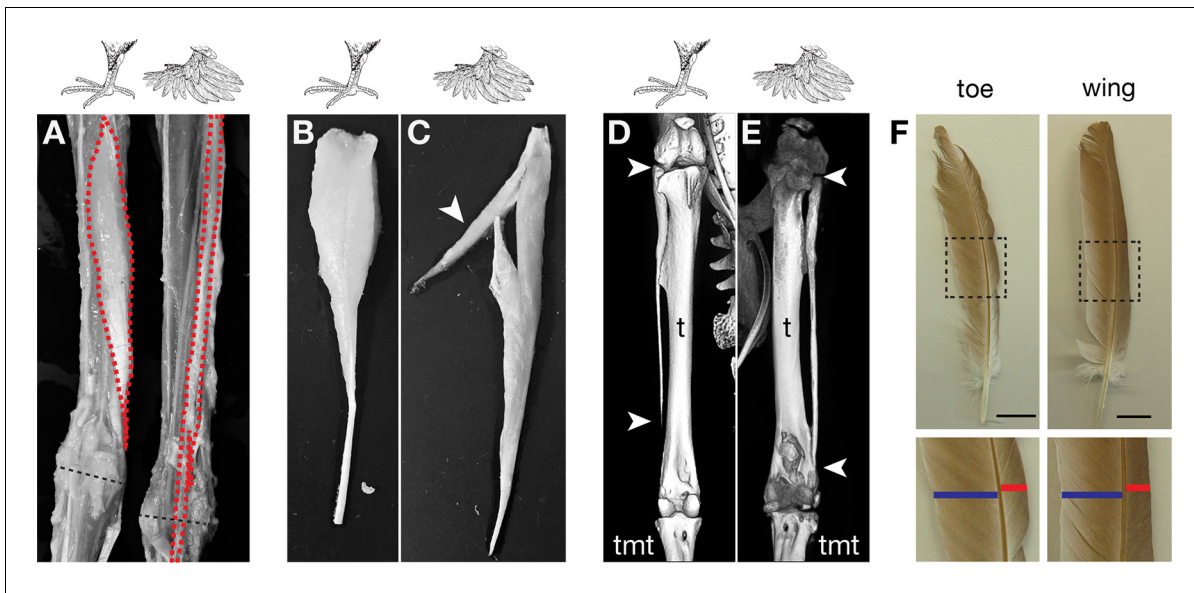


Figure 5. Muffed pigeons have re-patterned hindlimb musculoskeletal system and wing-like foot feathers. (A,B,C) Gross muscle morphology of scale-footed (feral) and muffed (Pomeranian pouter) left hindlimbs, dorsal view. (A) Skin and superficial muscles have been removed to reveal re-patterning of the fibularis longus (FL, red). Dashed black line, approximate position of ankle joint axis. (B,C) The FPP3 is a pinnate muscle in scale-footed pigeons (B), but a slip of fibers fuses with the adjacent FL in muffed pigeons (arrowhead in C). (D,E) X-ray computed tomography images of scale-footed (feral, right leg) and muffed (English trumpeter, left leg) hindlimbs. Arrowheads mark the proximal and distal ends of the fibula. The wild-type pigeon fibula (D) is short and splint-like. In the muffed bird (E), the fibula extends from the knee to the ankle. We observed distal elongation of the fibula in another muffed breed (fairy swallow) but the fibula did not completely extend to the ankle (not shown). t, tibia; tmt, tarsometatarsus. (F) Toe and wing (flight) feathers of a muffed pigeon (English trumpeter), highlighting vane width asymmetries. Blue bar, inner vane; red bar, outer vane. Scale bar = 2 cm.

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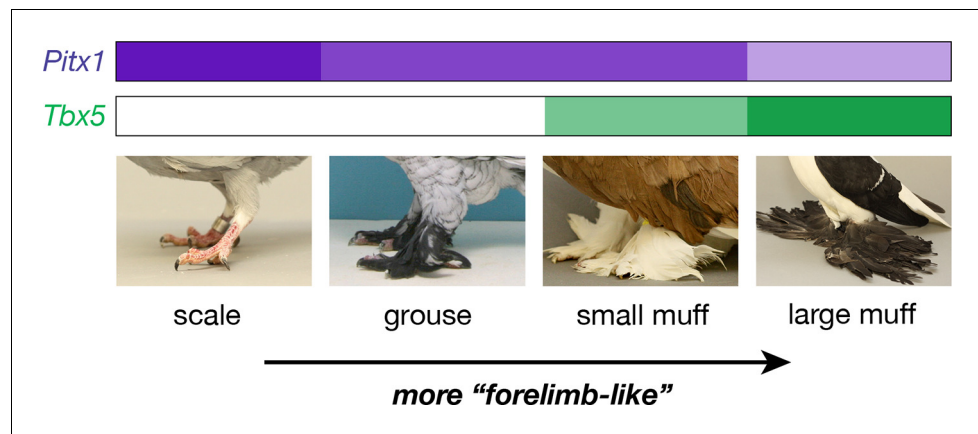


Figure 6. Model describing link between *Pitx1* and *Tbx5* expression levels and foot epidermal appendage morphology. Darker colors indicate higher expression levels. Decreased expression of *Pitx1* and ectopic expression of *Tbx5* are associated with foot feathering (and other morphological transformations) in domestic pigeons.

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