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Pigeon foot feathering reveals conserved limb identity networks

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A B S T R A C T

The tetrapod limb is a stunning example of evolutionary diversity, with dramatic variation not only among distantly related species, but also between the serially homologous forelimbs (FLs) and hindlimbs (HLs) within species. Despite this variation, highly conserved genetic and developmental programs underlie limb development and identity in all tetrapods, raising the question of how limb diversification is generated from a conserved toolkit. In some breeds of domestic pigeon, shifts in the expression of two conserved limb identity transcription factors, *PITX1* and *TBX5*, are associated with the formation of feathered HLs with partial FL identity. To determine how modulation of *PITX1* and *TBX5* expression affects downstream gene expression, we compared the transcriptomes of embryonic limb buds from pigeons with scaled and feathered HLs. We identified a set of differentially expressed genes enriched for genes encoding transcription factors, extracellular matrix proteins, and components of developmental signaling pathways with important roles in limb development. A subset of the genes that distinguish scaled and feathered HLs are also differentially expressed between FL and scaled HL buds in pigeons, pinpointing a set of gene expression changes downstream of *PITX1* and *TBX5* in the partial transformation from HL to FL identity. We extended our analyses by comparing pigeon limb bud transcriptomes to chicken, anole lizard, and mammalian datasets to identify deeply conserved *PITX1*- and *TBX5*-responsive components of the limb identity program. Our analyses reveal a suite of predominantly low-level gene expression changes that are conserved across amniotes to regulate the identity of morphologically distinct limbs.

1. Introduction

The tetrapod limb is an extraordinary example of morphological variation. Dramatic evolutionary diversification of limb form and function has enabled animals to thrive in diverse terrestrial, fossorial, aquatic, and aerial environments. The limb is unusual in that phenotypic variation characterizes not only limbs of different species, but also the serially homologous forelimbs (FLs) and hindlimbs (HLs) within a species. Limb variation between FLs and HLs is arguably most striking in birds, in which divergent patterning of the muscles, tendons, and bones that form the limb is coupled with variation in the epidermal appendages that cover each limb type. For example, the elaborately feathered epidermis of the avian wing is dramatically different from the scaled epidermis that typically covers the feet.

Despite highly divergent adult limb morphologies among and within species, limb patterning and morphogenesis is orchestrated by deeply conserved genetic and developmental programs. As a result of decades of elegant descriptive and experimental studies in canonical model organisms (especially mouse and chicken), the cellular, genetic, and molecular regulators of limb development are relatively well defined (reviewed in Petit et al., 2017; Tickle, 2015; Rabinowitz and Vokes, 2012; Duboc and Logan, 2011b; Towers and Tickle, 2009; Tickle, 2004). These studies reveal that the integration of several key developmental signaling pathways – including Fibroblast Growth Factor (FGF), Bone Morphogenetic Protein (BMP), Wnt, Sonic Hedgehog (SHH), and Retinoic Acid (RA) pathways – is required for proper limb bud position, initiation, outgrowth, and patterning.

In contrast, FL and HL identity is associated with the reciprocal expression patterns of a smaller set of genes, including the transcription factors (TFs) *TBX5* (FL-specific expression), *TBX4* (HL-specific), and *PITX1* (HL-specific) (Simon et al., 1997; Logan et al., 1998; Szeto et al., 1996; Gibson-Brown et al., 1996; Shang et al., 1997). Over the past 20 years, a combination of misexpression and genetic studies has illuminated essential roles for each of these TFs in limb development. The paralogs *TBX5* and *TBX4* direct the respective initiation of FL and HL bud development through activation of the FGF and Wnt signaling pathways (Takeuchi et al., 2003). In mice, $Tbx5^{-/-}$ mutants fail to form a FL bud, while $Tbx4^{-/-}$ mutants form a HL bud that arrests early in development due to a loss of *FGF10* expression maintenance (Agarwal et al., 2003; Takeuchi et al., 1999; Rallis et al., 2003; Naiche and Papaioannou, 2003, 2007). Similarly, loss of *tbx5* function in zebrafish results in a failure to form the pectoral fin (FL equivalent) (Ahn et al., 2002; Garrity et al., 2002), and disruption of *tbx4* nuclear localization in the naturally occurring *pelvic finless* zebrafish strain is associated with arrested pelvic

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fin (HL equivalent) development (Don et al., 2016). *Tbx5* and *Tbx4* are not essential for development of limb-specific identities in mice (Hasson et al., 2007; Naiche and Papaioannou, 2007; Minguillon et al., 2005); however, misexpression of *TBX4* in the FL or *TBX5* in the HL of chick embryos causes a partial transformation in limb identity in a subset of embryos, including changes in digit number, limb flexure, and epidermal appendage type (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Although the basis for the discrepancies between mouse and chick experiments remains unclear, it is possible that *TBX5* and *TBX4* have subtly different functions in mammals and birds, which diverged more than 300 million years ago (Horton et al., 2008).

Unlike *TBX4* and *TBX5*, *PITX1* is not required for limb bud initiation or outgrowth, but instead regulates these processes indirectly via activation of *TBX4* (Minguillon et al., 2005; Szeto et al., 1999; Logan and Tabin, 1999; Duboc and Logan, 2011a). In addition to *TBX4*-dependent regulation of HL outgrowth, *PITX1* serves a second essential role in limb development via *TBX4*-independent regulation of HL identity (Duboc and Logan, 2011a). In *Pitx1*$^{-/-}$ mutant mice, HLs develop, but lack HL-specific morphological characteristics (Szeto et al., 1999). In both chick and mouse embryos, ectopic expression of *PITX1* in the developing FL bud causes a partial transformation to a more HL-like identity (Delaurier et al., 2006; Logan and Tabin, 1999). Similarly, mutations at the human *PITX1* locus are associated with HL-like characteristics in the FL (AL-Qattan et al., 2013) and congenital HL defects (Gurnett et al., 2008). *PITX1* regulatory mutations have also been discovered in natural populations of three-spine stickleback fish, in which loss of *PITX1* gene expression contributes to the adaptive loss of pelvic (HL) structures (Shapiro et al., 2004; Chan et al., 2010).

Substantial research efforts over the past two decades have helped decipher the genetic networks regulated by *PITX1*, *TBX4*, and *TBX5* during limb development (reviewed in Duboc and Logan, 2011b; Rabinowitz and Vokes, 2012; Tickle, 2015; Petit et al., 2017). More recently, contemporary genomic approaches have opened new avenues for defining downstream targets in an unbiased manner. Particular progress has been made in defining the network downstream of *PITX1* by comparing limb buds from wild-type and *Pitx1*$^{-/-}$ mutant mice using massively parallel sequencing of both RNA transcripts to identify differentially-expressed genes and DNA regions captured by chromatin

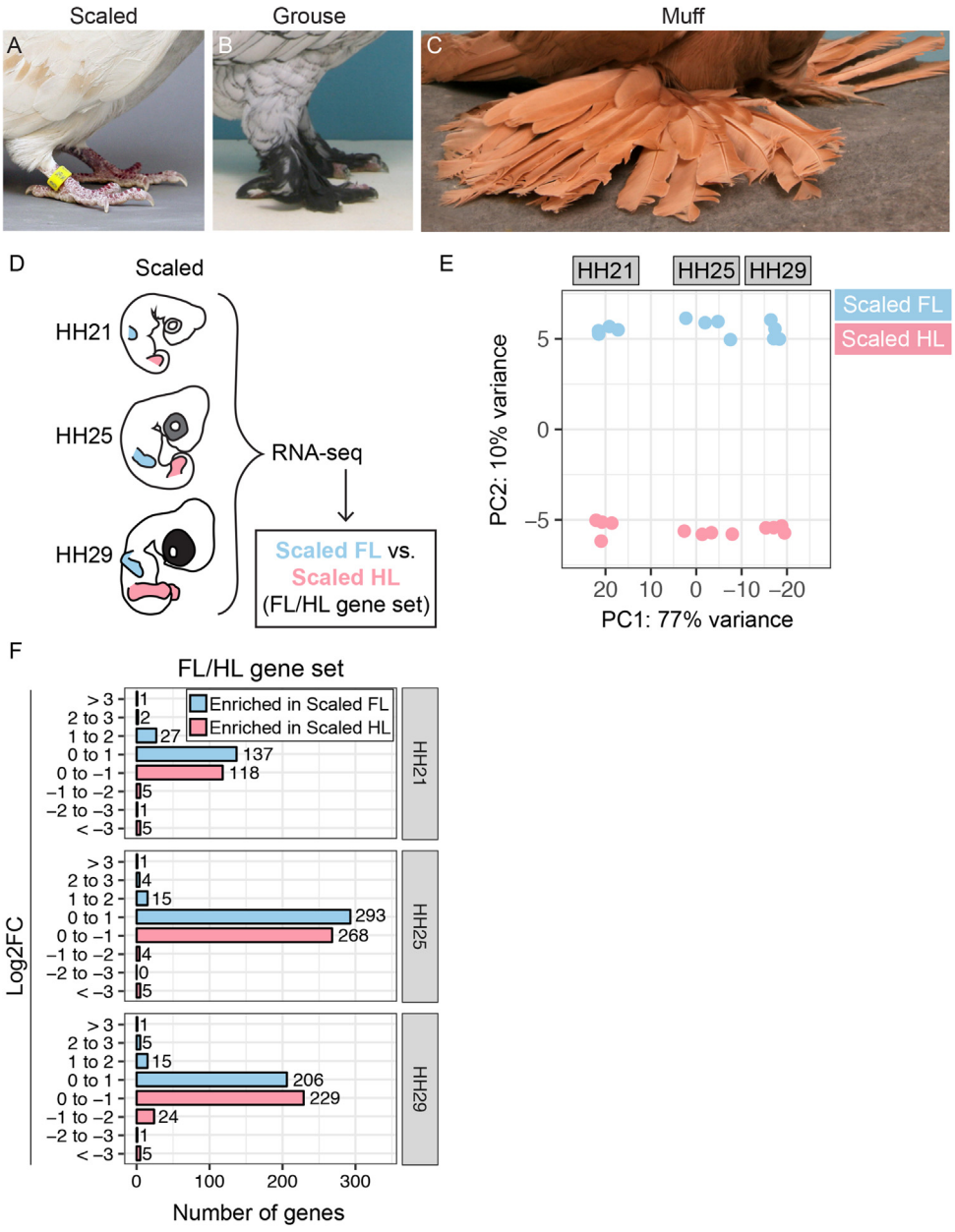


Fig. 1. Comparative transcriptomics to identify gene expression changes associated with pigeon limb identity. (**A-C**) Hindlimb phenotypes in domestic pigeon breeds, including scaled (A), grouse (B), and muff (C). (**D**) Experimental approach to identify differentially expressed genes at three stages (HH21, HH25, and HH29) of FL vs. HL development in pigeons with scaled feet. (**E**) PCA based on top 500 most variable genes in FL and scaled HLs. PC1 (77% of variance) separates samples by stage, PC2 (10% of variance) separates samples by limb type. (**F**) Number of genes that show FL-enriched or scaled HL-enriched expression pattern at HH21, HH25, or HH29. Genes are grouped based on Log2 fold change (FC). FL = forelimb; HL = hindlimb; HH = Hamburger-Hamilton stage. Photo credits: A, Sydney Stringham; B and C, Thomas Hellmann.

immunoprecipitation to locate putative regulatory regions (RNA-seq and ChIP-seq, respectively) (Nemec et al., 2017; Infante et al., 2013; Wang et al., 2018). These approaches have resulted in a more comprehensive view of *PITX1*-responsive gene networks (and could be used to similarly reveal *TBX5*-and *TBX4*-responsive networks). Despite these important advances, we still have a limited understanding of the transcriptomic changes that result from less dramatic (non-binary) shifts in gene expression that are associated with evolutionary diversification of limb morphology.

The domestic pigeon (*Columba livia*) is an outstanding model to study the evolution of genetic and developmental programs that underlie limb diversification. Pigeons display striking variation in HL morphology within a single species and are amenable to genetic crosses, genomic analyses, and embryonic studies (Shapiro et al., 2013; Shapiro and Domyan, 2013; Domyan and Shapiro, 2017). While most pigeons have scaled feet, in some breeds scaled epidermis is replaced by skin with a range of feather morphologies (Fig. 1A–C). Classical genetic experiments show that large feather "muffs" are caused by the synergistic effects of mutant alleles at two loci, *grouse* (*gr*) and *Slipper* (*Sl*), which independently produce smaller foot feathers (Doncaster, 1912; Wexelsen, 1934; Hollander, 1937; Levi, 1986). We recently showed that *gr* and *Sl* are *cis*-regulatory alleles of the limb identity genes *PITX1* and *TBX5*, respectively (Domyan et al., 2016), and proposed a model in which feathered feet are the result of a partial transformation from HL to FL identity. This genetically-encoded, partial transformation in identity provides a unique opportunity to determine how altered expression of conserved limb identity genes modifies the downstream HL identity transcriptional program.

The goal of this study is to identify genetic network changes that accompany shifts in limb identity. First, we identify FL- and HL-specific transcriptomic differences in pigeons. We then test for transcriptomic changes in pigeon HLs with partially altered limb identity attributable to changes in *PITX1* and *TBX5* regulation. Next, we compare our pigeon results with transcriptomic datasets from other amniotes to identify a set of genes whose expression typify FL and HL differences across broad phylogenetic distances. Finally, we find that a subset of the highly-conserved FL genetic program is also differentially expressed in mutant pigeon HLs with partial identity transformations.

2. Results

2.1. Gene expression changes associated with normal FL and HL development in pigeons

To identify differentially expressed transcripts associated with normal FL and HL development in pigeons, we performed RNA-seq on FL and HL buds from scale-footed pigeon embryos at three early stages of embryonic development (approximate pigeon equivalents of Hamburger-Hamilton (HH) stage 21, HH25, and HH29; Hamburger and Hamilton, 1951) (Fig. 1D). Principal component analysis (PCA) based on the 500 most differentially expressed genes revealed that PC1 accounts for 77% of sample variation and corresponds to developmental stage, while PC2 accounts for 10% of variation and separates samples by limb type (FL vs. HL) (Fig. 1E). Together, PC1 and PC2 group samples into six discrete clusters based on developmental stage and limb type.

We identified a set of 978 genes that are differentially expressed between FL and HL at HH21, HH25, and/or HH29 ("FL/HL" gene set; Table 1, Supplemental Fig. 1, and Supplemental Table 1). Within the FL/HL gene set, only 12 genes are strongly enriched (defined here as an absolute value Log2FC > 2) in a FL- or HL-specific manner (Fig. 1F), all of which are TFs that have been implicated in vertebrate limb development to varying degrees (Takeuchi et al., 1999; Rodriguez-Esteban et al., 1999; Szeto et al., 1999; Logan and Tabin, 1999; Zakany and Duboule, 2007; Tarchini et al., 2006; Capellini et al., 2006, 2010; Boulet and Capecchi, 2004; Wellik, 2003; Davis and Capecchi, 1994; Narkis et al., 2012; Feenstra et al., 2012). In contrast, the majority (86–95%) of differentially expressed genes changed less than 2-fold (Log2FC < 1) between FL and HL (Fig. 1F). These results suggest that, during early pigeon development, a small set of large gene expression changes is coupled with a large set of subtle gene expression changes to differentiate FL and HL identities. Notably, the number and level of gene expression changes that distinguish pigeon FL and HL is remarkably similar to what was recently reported for mouse FL and HL buds (Nemec et al., 2017), despite the radical differences between FL and HL morphology in pigeons and the more modest differences between limbs in mice.

2.2. Dynamic TBX5 and PITX1 expression differences characterize feathered HL development

We previously demonstrated that in pigeons, the grouse phenotype is associated with a *cis*-regulatory mutation that reduces *PITX1* expression in the HH25 HL, while the muff phenotype is associated with *cis*-regulatory mutations that cause a combination of *PITX1* reduction and ectopic *TBX5* expression in the HH25 HL (Domyan et al., 2016; Boer et al., 2017). To determine if *PITX1* and *TBX5* expression differ in scaled, grouse, and muff HLs at other stages of development, we measured *PITX1* and *TBX5* expression in HH21, HH25, and HH29 HL buds by quantitative reverse-transcriptase PCR (qRT-PCR) (Supplemental Fig. 2). We found that *TBX5* is ectopically expressed in muff HLs relative to scaled and grouse HLs at all stages analyzed. *PITX1* is significantly reduced in muff

Table 1
Summary of pigeon differential expression gene sets.

Category	Comparison	Differential expression gene sets		
		FL/HL	Grouse	Muff
Total	DE genes	978	1425	3202
	Shared between grouse and muff	–	42% (601/1425)	19% (601/3202)
	Shared with FL/HL	–	8% (108/1425)	10% (334/3202)
TFs	DE genes	122	50	105
	Shared between grouse and muff	–	42% (21/50)	20% (21/105)
	Shared with FL/HL	–	18% (9/50)	38% (40/105)
ECM	DE genes	81	35	92
	Shared between grouse and muff	–	43% (15/35)	16% (15/92)
	Shared with FL/HL	–	26% (9/35)	29% (27/92)

Differential expression gene sets: FL/HL, FL vs. HL from scale-footed pigeons; grouse, grouse HL vs. scaled HL; muff, muff HL vs. scaled HL. Total, total number of significantly differentially expressed genes in each gene set; TFs, transcription factors; ECM, extracellular matrix genes; DE, differentially expressed. Percentage values indicate overlap of two gene sets. For example, of the 1425 genes DE in the grouse (grouse HL vs. scaled HL) comparison, 601/1425 (42%) are also DE in the muff (muff HL vs. scaled HL) comparison, and 108/1425 (8%) are also DE in the FL/HL (FL vs. scaled HL) comparison.

HLs relative to scaled HLs at all stages analyzed (Supplemental Fig. 2). In contrast, *PITX1* is significantly reduced in grouse HLs relative to scaled HLs only at HH25 and HH29 (Supplemental Fig. 2), suggesting that the temporal dynamics of *PITX1* HL expression is different between grouse and muff pigeon breeds.

2.3. *PITX1-and TBX5-dependent gene expression during feathered HL development*

We predicted that the divergent *PITX1* and *TBX5* expression profiles during development of scaled, grouse, and muff HLs could reveal gene expression changes downstream of *TBX5* and/or *PITX1* that are associated with limb diversification. To test this hypothesis, we performed RNA-seq on limb buds from grouse and muff embryos isolated at HH21, HH25, and HH29 (Fig. 2A). We reasoned that comparison of differentially expressed gene sets from scaled vs. grouse HLs ("grouse" gene set) and scaled vs. muff HLs ("muff" gene set) would enable us to distinguish *PITX1*-responsive gene expression changes (misexpressed in both grouse and muff HLs) from the effects of *TBX5* and/or *PITX1* + *TBX5* co-regulation (misexpressed only in muff HLs).

To test for consistency with our qRT-PCR results, we first examined the expression profiles of *PITX1* and *TBX5* in the RNA-seq samples from scaled, grouse, and muff limb buds. As expected, *TBX5* is highly expressed in all FL samples at all developmental stages with no significant difference in expression among FLs from scaled, grouse, or muff embryos (Supplemental Fig. 3). Within HL samples, *TBX5* is significantly enriched in muff HLs relative to scaled and grouse HLs at all stages (Supplemental Fig. 3). *PITX1*, which is expressed only in HLs, is significantly reduced in muff HLs relative to scaled HLs at all stages (Supplemental Fig. 4). In contrast, *PITX1* expression is reduced in grouse HLs relative to scaled HLs only at HH25 and HH29 (Supplemental Fig. 4). Thus, our analyses of *PITX1* and *TBX5* expression by qRT-PCR and RNA-seq yielded qualitatively identical results.

To determine the relationship between scaled, grouse, and muff limb bud transcriptomes, we performed PCA as described above for limb buds from scale-footed pigeons. Addition of the grouse and muff datasets had a minimal effect on the overall structure of the results; FL and HL samples remain clustered by limb type and developmental stage (Fig. 2B and Supplemental Fig. 5). Within each HL cluster, muff HLs separated slightly from scaled and grouse HLs and clustered closer to their respective stage-

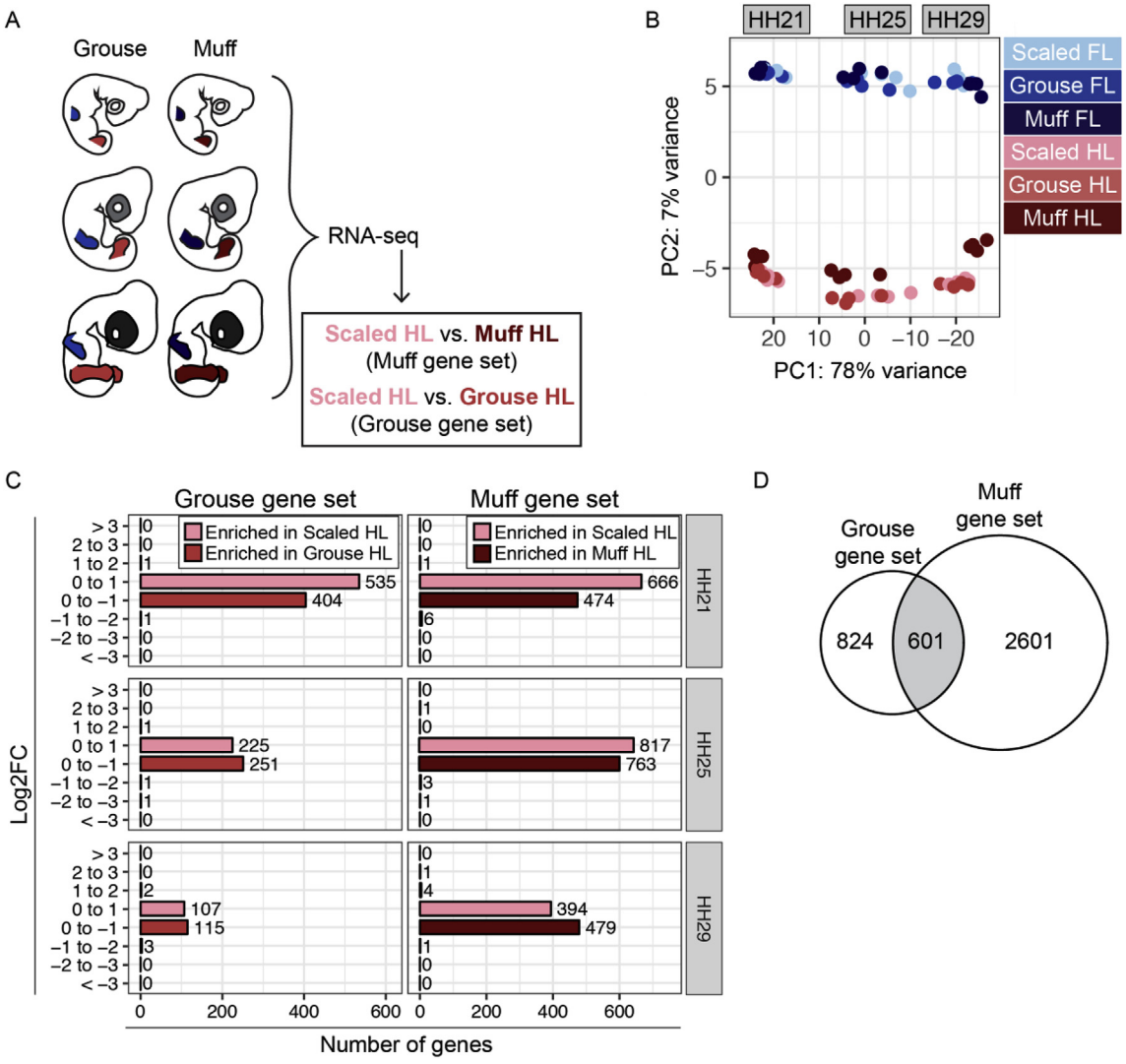


Fig. 2. Comparison of hindlimb transcriptomes from scaled, grouse, and muff pigeon embryos. (**A**) Experimental approach to identify differentially expressed genes from grouse and muff limb buds at HH21, HH25, and HH29. (**B**) PCA based on top 500 most variable genes from all FL and HL limb bud samples. PC1 (78% of variance) separates samples by developmental stage, PC2 (7% of variance) separates samples by FL vs. HL identity. (**C**) Number of genes enriched in scaled HL or feathered HL specific manner at HH21, HH25, or HH29. Genes are grouped based on Log2FC from scaled HL vs. grouse HL or scaled HL vs. muff HL comparisons. (**D**) Venn diagram comparing differentially expressed gene sets from scaled HL vs. grouse HL and scaled HL vs. muff HL comparisons (intersection p-value = 4.3e-83).

matched FL clusters on PC2.

Next, we performed differential expression analyses to identify transcriptional changes associated with altered *PITX1* and/or *TBX5* expression in feathered HLs. Relative to scaled HLs, a total of 1425 genes are misexpressed in grouse HLs (grouse gene set) and 3202 genes are misexpressed in muff HLs (muff gene set) at HH21, HH25, and/or HH29 (Table 1, Supplemental Fig. 6, and Supplemental Tables 2–3). Similar to the FL vs. scaled HL differential expression results, feathered HL development is characterized by extensive low-level gene expression changes, with nearly all differentially expressed genes (>99%) altered by less than 2-fold (Log2FC < 1, Fig. 2C).

By comparing the grouse and muff gene sets, we identified 601 genes that are putatively *PITX1*-responsive, as they are misexpressed in both grouse and muff HLs (intersection p-value = 4.3e-83; Fig. 2D, Table 1, and Supplemental Table 4). A subset (125/601) of the *PITX1*-responsive genes were also previously identified in transcriptomic analyses of mouse *Pitx1*$^{-/-}$ HLs (Wang et al., 2018; Nemec et al., 2017), including the TFs *EMX2, HOXD11, RFX4*, *TBX15,* and *ZEB2.* Other *PITX1*-responsive genes encode components of several important developmental signaling pathways, as well as a variety of extracellular matrix (ECM) proteins (see below). These independently identified pigeon and mouse *PITX1*-responsive genes show a significant degree of overlap (intersection p-value = 3.7e-05; Supplemental Fig. 7 and Supplemental Table 4). The *PITX1*-responsive gene set also includes genes with known roles in limb development such as *short stature homeobox* (*SHOX*) (Decker et al., 2011; Tiecke et al., 2006) that, to our knowledge, were not known to be regulated by *PITX1*. Notably, *Shox* is not present in the mouse genome (but is present in other mammalian genomes, including human), precluding the identification of *Shox* as a *Pitx1* target in previous genomic analyses of mouse *Pitx1*$^{-/-}$ HLs (Wang et al., 2018; Nemec et al., 2017).

In addition to the *PITX1*-responsive genes that are misexpressed in both grouse and muff pigeon HLs, we identified 824 genes that are misexpressed only in grouse HLs (Fig. 2D). This finding raises the possibility that either (i) distinct genetic programs are activated depending on *PITX1* expression level (*PITX1* is differentially expressed between grouse and muff HLs; Supplemental Fig. 4), (ii) additional genetic changes modify the grouse phenotype but were not detected in our whole-genome scans for allele frequency differentiation (Domyan et al., 2016), and/or (iii) there are breed-specific gene expression changes that distinguish Oriental Frills (grouse) from Racing Homers (scaled) and English Trumpeters (muff) and are unrelated to HL phenotypes. We also identified 2601 genes that are misexpressed only in muff HLs (Fig. 2D). These genes are likely regulated by *TBX5*, although we cannot rule out co-regulation by *TBX5* and *PITX1*. In summary, by comparing the transcriptional profiles associated with scaled, grouse, and muff HL development, we discovered sets of genes that are putative targets of *PITX1* and/or *TBX5* during pigeon limb development. While some of the genes we identified are known targets of *PITX1* or *TBX5*, others were not previously linked to *PITX1*, *TBX5,* or limb development.

2.4. Partial co-option of pigeon FL genetic program during feathered HL development

We next wanted to know if feathered HL development is accompanied by FL-like transcriptional changes in genes other than *PITX1* and *TBX5*. Therefore, we probed the grouse and muff gene sets for genes that are also differentially expressed between FL and scaled HL (i.e., in the FL/HL gene set).

We first identified signatures of FL identity associated with reduced *PITX1* expression by comparing the grouse and FL/HL gene sets. Of the genes that are misexpressed in grouse HLs, ~8% (108/1425) are also differentially expressed between FL and scaled HL (intersection p-value = 0.029; Fig. 3A, Table 1, and Supplemental Table 2). Within this set of 108 genes, we identified two categories of genes with FL-like gene expression patterns: FL-like Group 1 genes are enriched in FLs and grouse HLs relative to scaled HLs, while FL-like Group 2 genes have reduced expression in FLs and grouse HLs relative to scaled HLs (Fig. 3B–D). FL-like Group 1 includes the TFs *HOXD11* and *LMX2*, as well as *DUSP6*, a negative regulator of FGF signaling (Fig. 3B and C). FL-like Group 2 includes *PITX1*, other TFs such as *VSX1* and *RFX4*, *BMP5*, and several genes that encode cell adhesion and cytoskeletal proteins (including *LVRN*, *DSC1*, *VCAM1*, and *SPTBN5*) (Fig. 3B,D).

We next identified signatures of FL identity that are putatively regulated by *TBX5* (or co-regulated by *TBX5* and *PITX1*) by comparing the muff and FL/HL gene sets. Of the genes that are misexpressed in muff HLs, ~10% (334/3202) are also differentially expressed between FL and scaled HL (intersection p-value = 7.5e-24; Fig. 4A, Table 1, and Supplemental Table 3). Similar to the grouse gene set, we identified two categories of genes with FL-like gene expression patterns (Fig. 4B–D). FL-like Group 1 (enriched in FL and muff HL relative to scaled HL) includes the TFs *TBX5*, *HOXD11*, *GBX2*, *ALX1*, and *EMX2*, as well as the Wnt-associated scaffolding gene *DAAM1* (Fig. 4B and C). FL-like Group 2 (reduced in FL and muff HL relative to scaled HL) includes the TFs *PITX1*, *PAX9*, *VSX1*, *RFX4*, *NKX3-2*, *DACH2* and *ZIC3*; components of developmental signaling pathways with known roles in limb development such as *WNT16*, *RDH10* and *BMP5*; and several genes encoding ECM proteins (Fig. 4B,D). In addition, many other genes display non-significant FL-like expression patterns in muff HLs, including the TFs *ISL1, SOX2, HOXB5, HOXD3, HOXD4, HOXC8, HOXD8, HOXD9, HOXD10,* and *HOXD11* (Fig. 4B,D).

2.5. TFs are differentially expressed in feathered HLs

In our analyses of the grouse and muff gene sets, we noted an enrichment of FL-like expression of genes encoding TFs, ECM proteins, and components of developmental signaling pathways. Hence, these gene classes could be key parts of the co-opted FL limb identity program in feathered HLs. To test this prediction, we next focused in on differentially expressed genes encoding TFs, ECM proteins, and developmental signaling components.

To identify TFs that are normally differentially expressed between FL and HL, we compared the FL/HL gene set to a genome-wide list of 1473 TFs (AnimalTFDB 2.0 database; Zhang et al., 2015). From this list, 122 TFs are differentially expressed between FLs and scaled HLs (Fig. 5A and Table 1). As expected, we detected strong FL enrichment of many known FL-specific TFs, including *TBX5, HOXD10, HOXD8, HOXD9,* and *ALX1* (Fig. 5A). Likewise, we detected HL enrichment of known HL-specific TFs, including *TBX4, PITX1, HOXC11, HOXC10*, *HOXB9,* and *ISL1* (Fig. 5A). Many other TFs showed FL or HL expression bias to a lesser degree, and some TFs displayed temporally dynamic FL/HL expression patterns (Fig. 5A).

We next identified TFs that are misexpressed during feathered HL development by comparing the grouse and muff gene sets to the genome-wide TF list (Fig. 5B and C). We found 50 TFs that are misexpressed in grouse HLs and 105 TFs that are misexpressed in muff HLs (Fig. 5B and C and Table 1). Comparison of the grouse and muff results revealed 21 TFs that are likely regulated by *PITX1*, as they are misexpressed in both grouse and muff HLs (Fig. 5B–D and Table 1). A subset of the TFs in the grouse and muff gene sets were also identified in the FL/HL gene set, suggesting that these TFs are co-opted from the normal FL identity program (Fig. 5B–D and Table 1). In addition, we found a set of TFs, including several HOX and T-box genes (*HOXA3, HOXA5, HOXA6, TBX15, IRX2*), that are misexpressed in feathered HLs but are not differentially expressed between FL and scaled HLs (Fig. 5B–D). These results suggest that, in addition to a partial redeployment of the FL identity program, feathered HL development involves the activation of TFs not normally required for differentiation of FLs and scaled HLs.

2.6. Differentially expressed ECM components in feathered HLs

In birds, ECM composition differs between scaled and feathered epidermis (Sengel, 1990). Consistent with this observation, recent

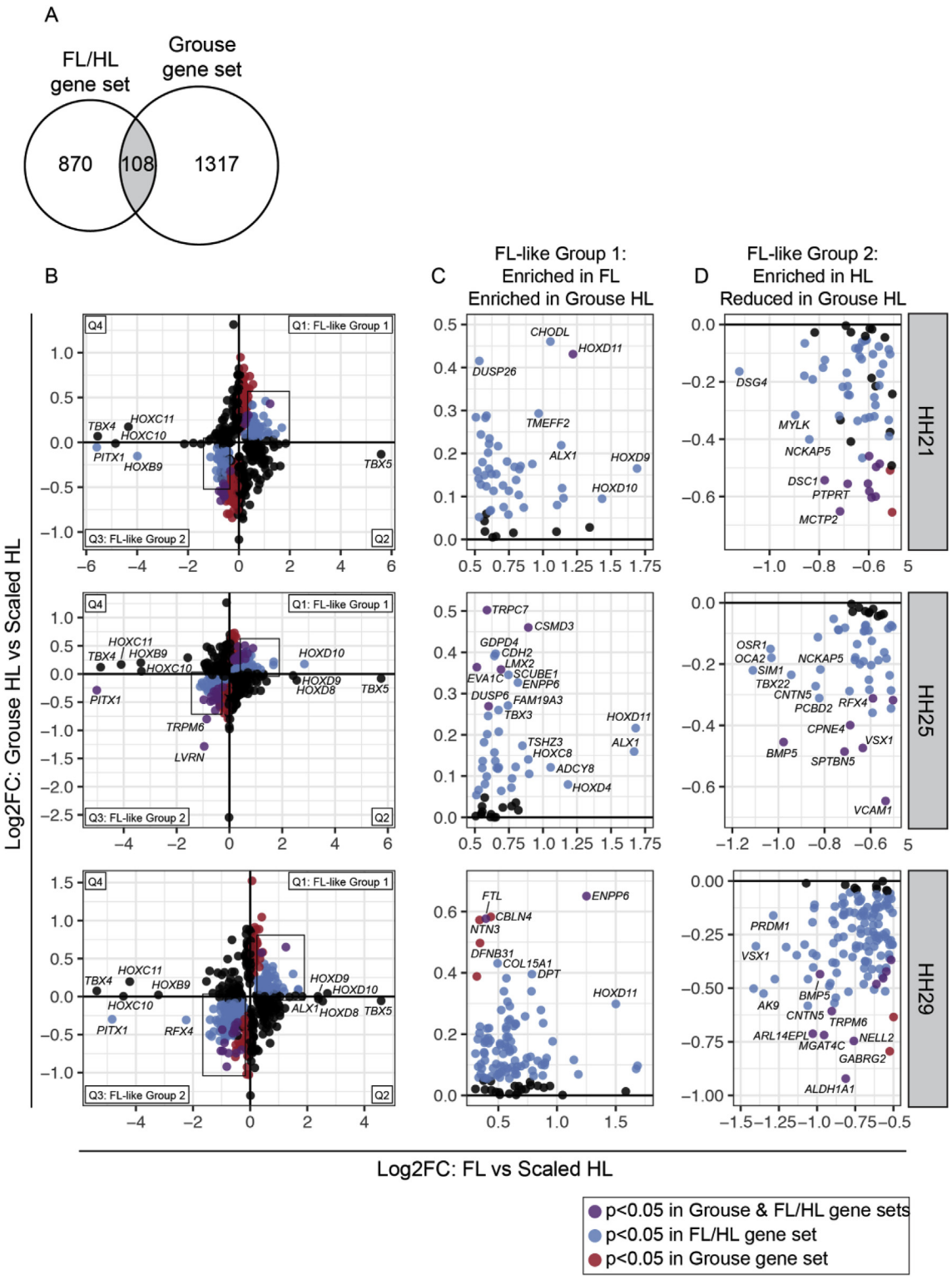


Fig. 3. A subset of genes in the grouse HL transcriptome display FL-like expression patterns. (**A**) Venn diagram comparing differentially expressed gene sets from FL vs. scaled HL and scaled HL vs. grouse HL comparisons (intersection p-value = 0.029). (**B**) Scatterplots of Log2FC values for all genes that are differentially expressed in FL vs. scaled HL or scaled HL vs. grouse HL at HH21, HH25, or HH29. Scatterplots are divided into four quadrants (Q); Q1 and Q3 highlight genes that show FL-like expression pattern in grouse HLs. Genes in Q1 (FL-like Group 1) and Q3 (FL-like Group 2) are color coded: blue dots denote genes that are significantly differentially expressed between FL and scaled HL; red dots denote genes that are significantly differentially expressed between scaled HL and grouse HL; purple dots represent genes that are significantly differentially expressed in both comparisons. Boxes indicate regions of scatterplot displayed in (C) or (D). (**C**) Detail of FL-like Group 1 genes. (**D**) Detail of FL-like Group 2 genes. Statistical significance: adjusted p-value < 0.05.

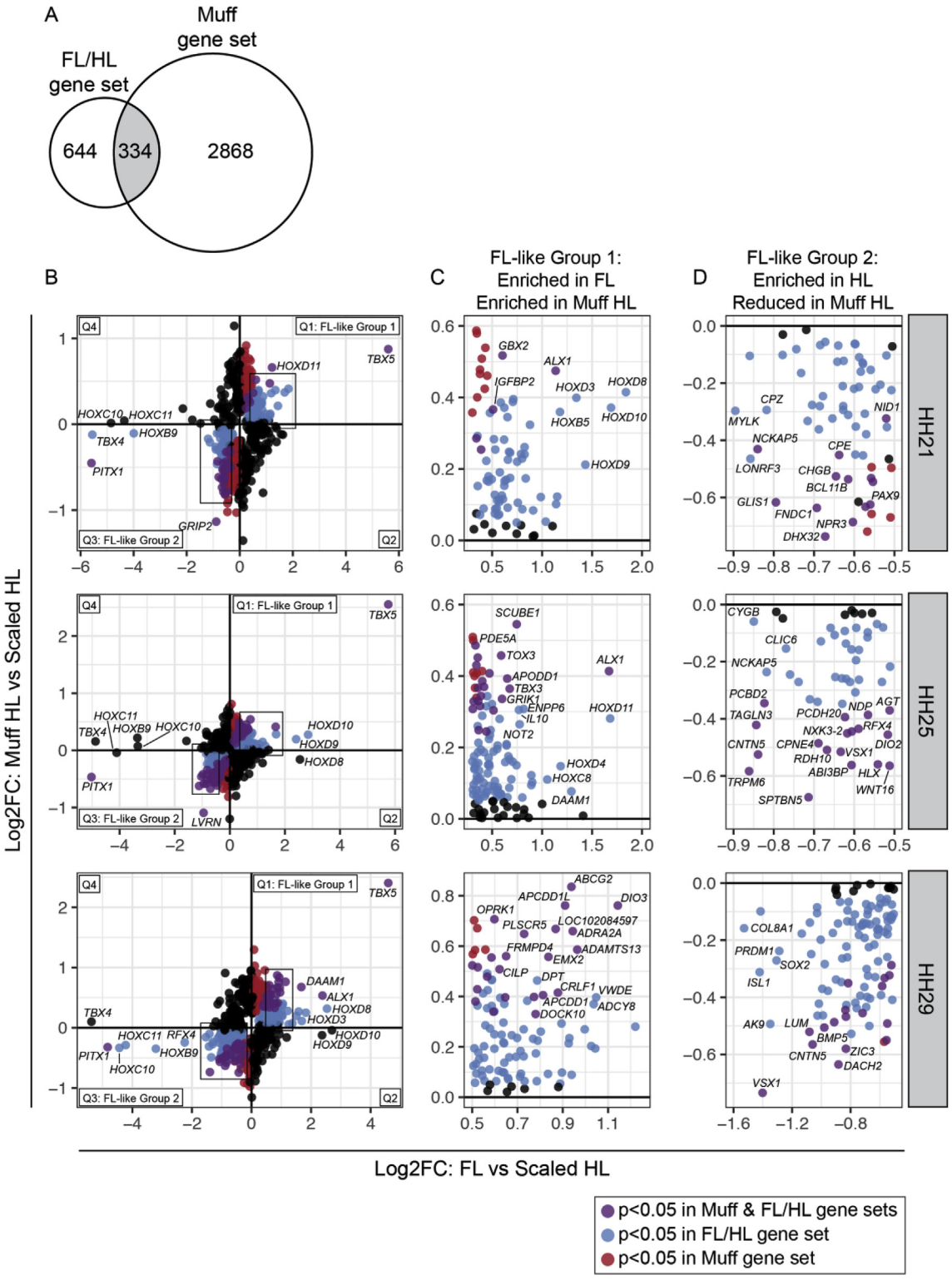


Fig. 4. A subset of genes in the muff HL transcriptome display FL-like expression patterns. (**A**) Venn diagram comparing differentially expressed gene sets from FL vs. scaled HL and scaled HL vs. muff HL comparisons (intersection p-value = 7.5e-24). (**B**) Scatterplots of Log2FC values for all genes that are differentially expressed in FL vs. scaled HL or scaled HL vs. muff HL at HH21, HH25, or HH29. Scatterplots are divided into four quadrants (Q); Q1 and Q3 highlight genes that show FL-like expression pattern in muff HLs. Genes in Q1 (FL-like Group 1) and Q3 (FL-like Group 2) are color coded: blue dots denote genes that are significantly differentially expressed between FL and scaled HL; red dots denote genes that are significantly differentially expressed between scaled HL and muff HL; purple dots represent genes that are significantly differentially expressed in both comparisons. Boxes indicate regions of scatterplot displayed in (C) or (D). (**C**) Detail of FL-like Group 1 genes. (**D**) Detail of FL-like Group 2 genes. Statistical significance: adjusted p-value < 0.05.

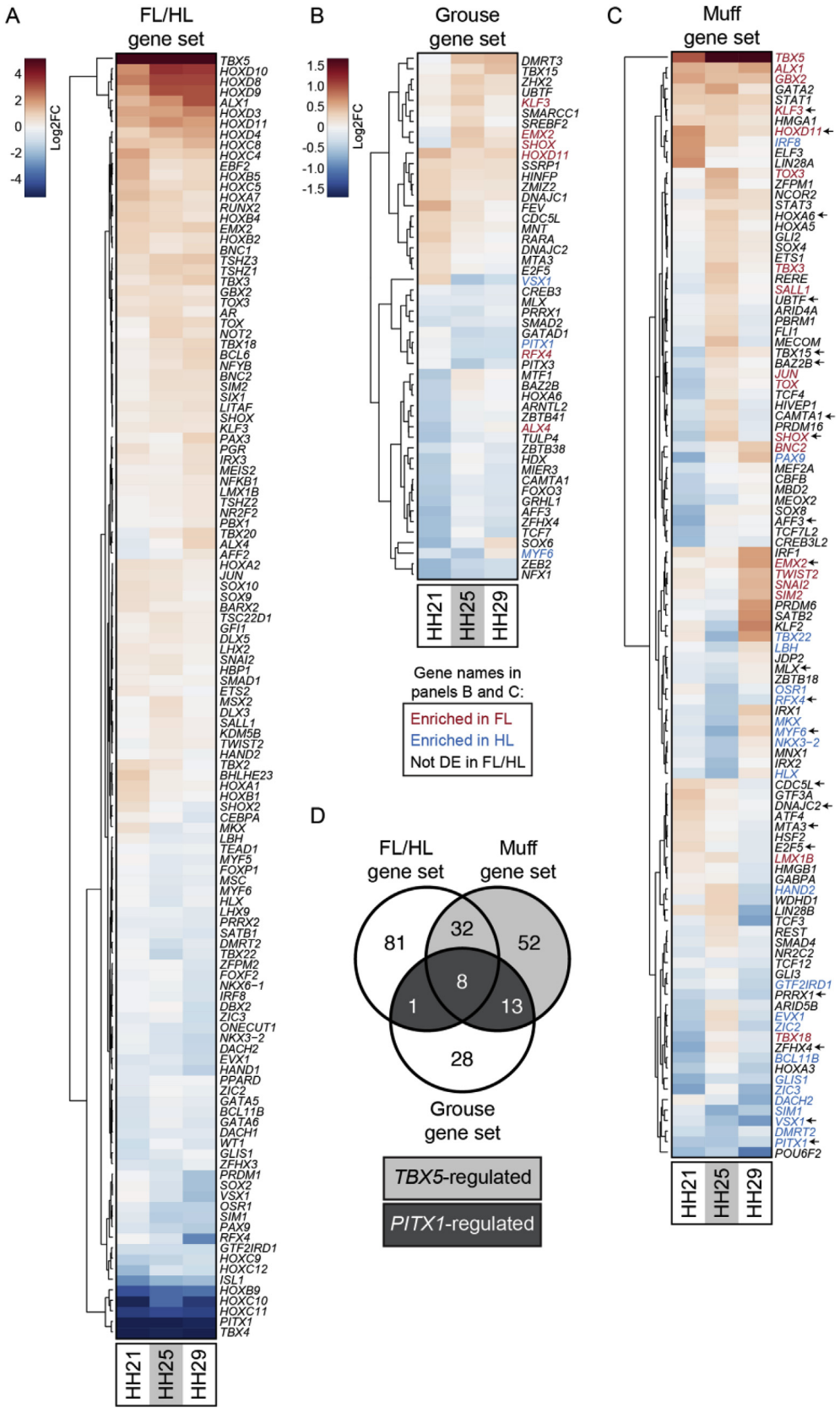


Fig. 5. Differential expression of transcription factors associated with pigeon limb identity. (**A-C**) Gene-wise hierarchical clustering heat maps of transcription factors (TFs) that are significantly differentially expressed between FL vs. scaled HL (A), scaled HL vs. grouse HL (B), or scaled HL vs. muff HL (C). For (A–C), all TFs that are significantly differentially expressed at one or more stages are included in the heat map. The scale for (A) is Log2FC −5 (HL-enriched) to 5 (FL-enriched); the scale for (B) and (C) is Log2FC −1.5 (scaled HL-enriched) to 1.5 (grouse HL-enriched). In (B) and (C), gene names highlighted in red or blue denote genes from FL vs. scaled HL comparison that are significantly FL-enriched or HL-enriched, respectively. In (C), arrows next to gene names highlight genes that are differentially expressed in both grouse and muff HLs relative to scaled HLs. (**D**) Venn diagram showing overlap of differentially expressed TFs from FL vs. scaled HL, scaled HL vs. grouse HL, and scaled HL vs. muff HL comparisons. Dark gray shading highlights TFs that are differentially expressed in all three comparisons and are putatively regulated by *PITX1*. Light gray shading denotes TFs that are differentially expressed only in FL vs. scaled HL and scaled HL vs. muff HL comparisons, suggesting regulation by *TBX5*.

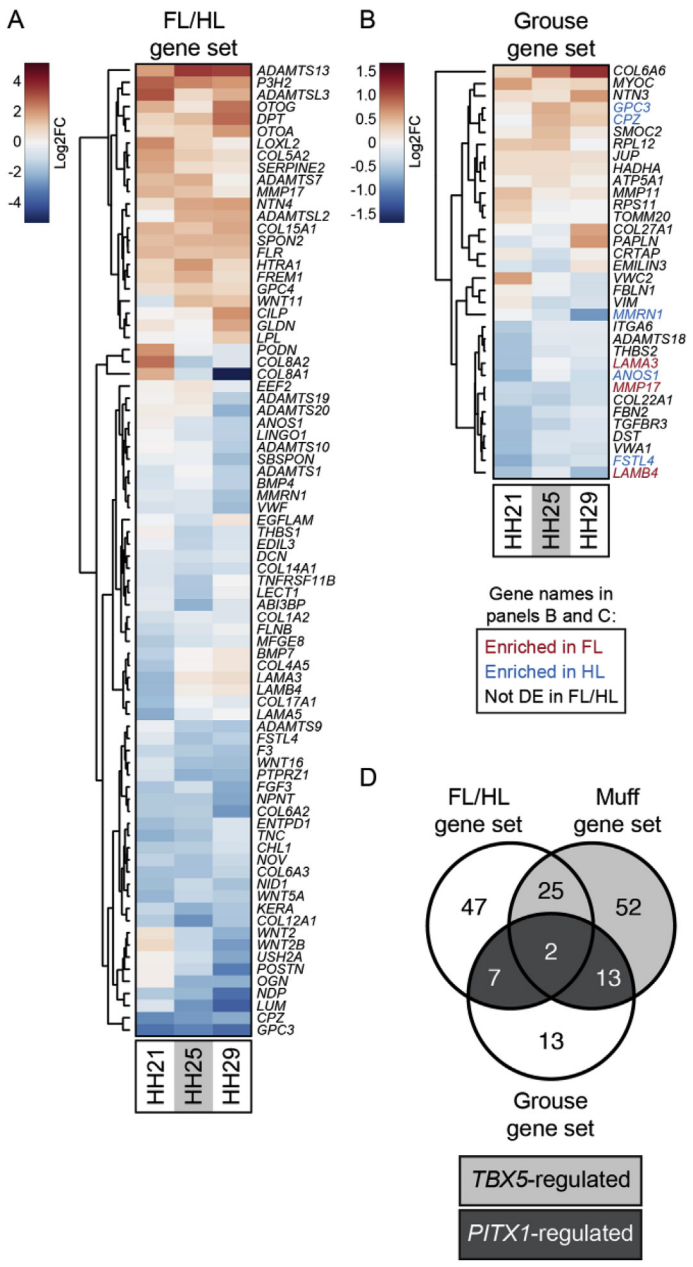


Fig. 6. Differential expression of genes encoding extracellular matrix components is associated with pigeon limb identity. (**A-C**) Gene-wise hierarchical clustering heat maps of genes encoding extracellular matrix (ECM) components that are significantly differentially expressed between FL vs. scaled HL (A), scaled HL vs. grouse HL (B), or scaled HL vs. muff HL (C). For (A–C), all ECM-encoding genes that are significantly differentially expressed at one or more stages are included in the heat map. The scale for (A) is Log2FC −5 (HL-enriched) to 5 (FL-enriched); the scale for (B) and (C) is Log2FC −1.5 (scaled HL-enriched) to 1.5 (muff HL-enriched). In (B) and (C), gene names highlighted in red or blue denote genes from FL vs. scaled HL comparison that are significantly FL-enriched or HL-enriched, respectively. In (C), arrows next to gene names highlight genes that are differentially expressed in both grouse and muff HLs relative to scaled HLs. (**D**) Venn diagram showing overlap of differentially expressed ECM-encoding genes from FL vs. scaled HL, scaled HL vs. grouse HL, and scaled HL vs. muff HL comparisons. Dark gray shading highlights ECM-encoding genes that are differentially expressed in all three comparisons and are putatively regulated by *PITX1*. Light gray shading denotes ECM-encoding genes that are differentially expressed only in FL vs. scaled HL and scaled HL vs. muff HL comparisons, suggesting regulation by *TBX5*.

transcriptomic analyses have identified specific ECM genes that are differentially expressed between these epidermal types in developing archosaur embryos (Wu et al., 2018; Musser et al., 2018; Lai et al., 2018). With the importance of the ECM in mind, we identified ECM genes putatively regulated by *PITX1* and/or *TBX5* during pigeon limb development by cross-referencing our FL/HL, grouse, and muff gene sets to a list of 491 genes annotated as ECM components in the Gene Ontology database (GO:0031012) (Ashburner et al., 2000; The Gene Ontology Consortium, 2017; Carbon et al., 2009). From this list, we identified 81, 35, and 92 ECM genes in the FL/HL, grouse, and muff gene sets, respectively (Fig. 6A–D and Table 1). By comparing the ECM genes in the grouse and muff gene sets, we found ECM genes that are downstream of *PITX1* and/or *TBX5* during early pigeon limb development, including ADAMTS proteases, collagens, and Wnt pathway ligands (Fig. 6D). Notably, almost half (12/27) of the ECM genes shared between the FL/HL, grouse, and muff gene sets were also found to be differentially expressed between feather- and scale-forming epidermis in chick embryos (Wu et al., 2018), supporting the model that distinct ECM signatures accompany the development of divergent limb identities in birds.

2.7. Modulation of developmental signaling pathways in feathered HLs

Normal limb development requires the integration of several key developmental signaling pathways including the SHH, FGF, BMP, RA, and Wnt pathways (reviewed in Duboc and Logan, 2011b; Tickle, 2015). Within the FL/HL gene set, we identified suites of genes involved in each of these pathways using Ingenuity Pathway Analysis (IPA) (Supplemental Figs. 8–11), suggesting that fine-tuning of developmental pathways is a normal part of FL vs. HL development. To determine if misexpression of *PITX1* and/or *TBX5* in feathered HLs is associated with altered expression of genes in these same pathways, we also probed the grouse and muff gene sets using IPA. In both the grouse and muff gene sets, we detected differential expression of genes associated with FGF, RA, and Wnt signaling (Supplemental Figs. 8–10), raising the possibility that *TBX5* and/or *PITX1* interacts with each of these pathways. We also identified a small number of genes within the muff gene set that are associated with BMP and SHH signaling, suggesting that *TBX5* may also interact with these pathways during early stages of limb development (Supplemental Fig. 11). Together, these results suggest that fine-tuning of

developmental signaling pathways by *PITX1* and *TBX5* is associated with the development of distinct limb morphologies, and provide a foundation for further investigation of interactions between *PITX1*, *TBX5*, and signaling pathways involved in limb development.

2.8. Evidence for conservation of limb identity programs among birds

Despite highly variable adult limb morphologies, the genetic toolkit that regulates limb development among vertebrates is evolutionarily conserved. However, the degree to which regulation of these genes is conserved across distantly related species remains unclear. To determine if limb-specific transcriptional profiles are conserved across large phylogenetic distances, we first compared gene expression in limb buds from pigeons and chickens, two distantly related avian species. We generated an RNA-seq dataset from FL and HL buds from scale-footed chicken embryos at developmental stages comparable to our pigeon dataset (HH21, HH25, and HH29). Consistent with the pigeon dataset, PCA based on the 500 most differentially expressed genes separated samples by stage (PC1, 81% of variance explained) and limb type (PC2, 10%) (Fig. 7A and Supplemental Fig. 5). We identified a set of 2160 genes

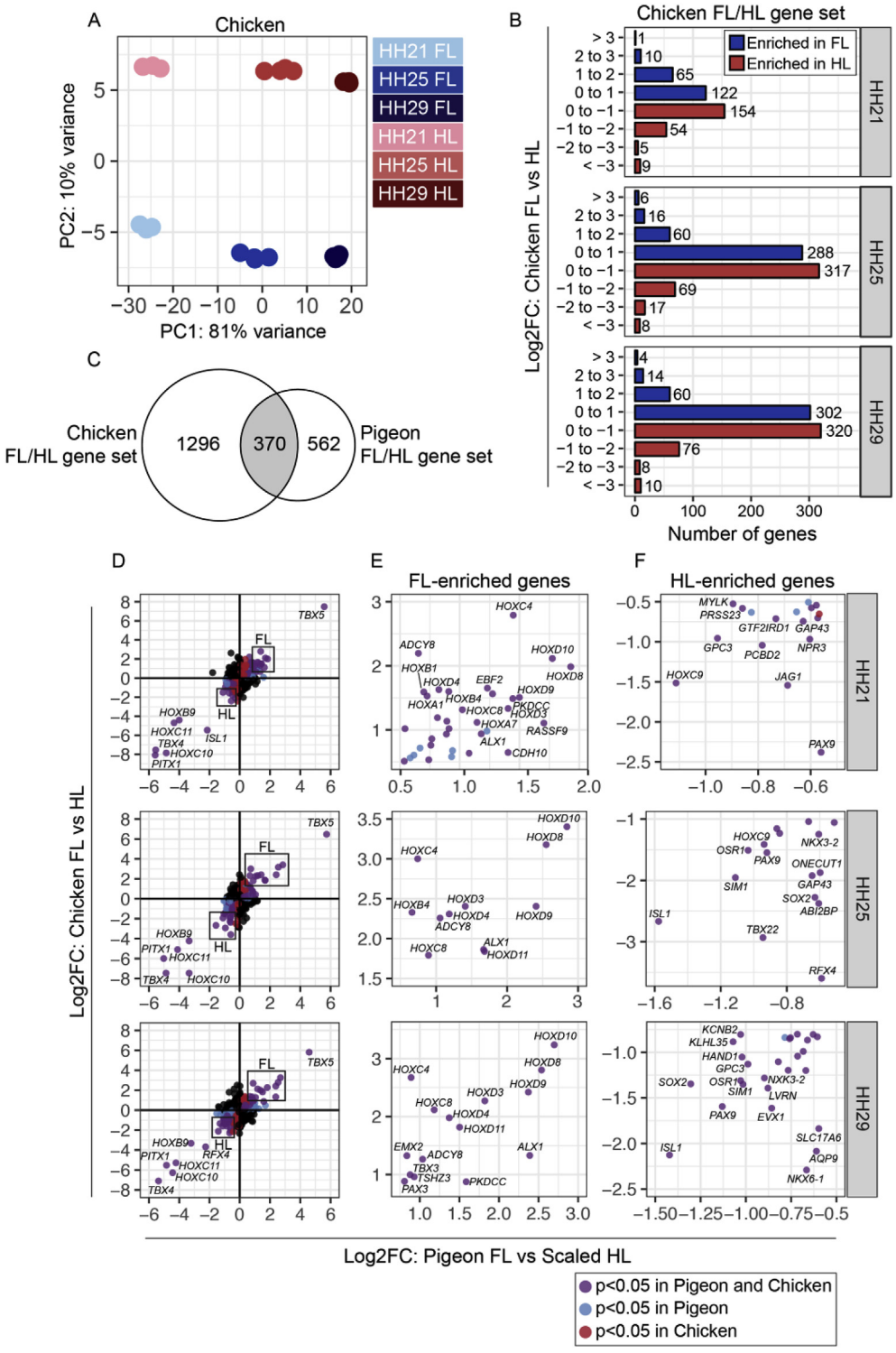


Fig. 7. Identification of conserved gene expression patterns in embryonic limb buds from chicken and pigeon. (**A**) PCA based on top 500 most variable genes in chicken FL and scaled HL buds at HH21, HH25, and HH29. PC1 (82% of variance) separates samples by stage; PC2 (11% of variance) separates samples by limb type. (**B**) Number of genes that show FL-enriched or scaled HL-enriched expression pattern in chicken embryos at HH21, HH25, or HH29. Genes are grouped based on Log2FC. (**C**) Venn diagram showing overlap of chicken FL vs. HL and pigeon FL vs. scaled HL gene sets (intersection p-value = 3.1e-61). (**D**) Scatterplots of Log2FC values for all genes that are differentially expressed in chicken FL vs. HL or pigeon FL vs. scaled HL at HH21, HH25, or HH29. Scatterplots are divided into four quadrants (Q); Q1 and Q3 highlight genes that show FL-enriched or HL-enriched expression in both avian species. Genes in Q1 (FL-enriched) and Q3 (HL-enriched) are color coded: blue dots denote genes that are significantly differentially expressed in pigeon FL vs. scaled HL; red dots denote genes that are significantly differentially expressed in chicken FL vs. HL; purple dots represent genes that are significantly differentially expressed in both comparisons. Boxes indicate regions of scatterplot displayed in (C) or (D). (**E**) Detail of FL-enriched genes. (**F**) Detail of HL-enriched genes. Statistical significance: adjusted p-value < 0.05.

that are differentially expressed between chicken FL and HL buds at HH21, HH25, and/or HH29 (chicken FL/HL gene set, Supplemental Fig. 12 and Supplemental Table 5). As in our pigeon analyses, we found that most of the gene expression changes between chicken FL and HL are relatively modest (Log2FC < 1) (Fig. 7B). Similar to our analysis of pigeon FL and HL buds, we identified a suite of TFs, genes encoding ECM proteins, and components of developmental signaling pathways that are differentially expressed between chicken FL and HL buds (Supplemental Fig. 13).

To determine the degree to which the limb identity network is conserved between pigeons and chickens, we compared the FL/HL gene sets from each species. For this analysis, we included only the genes that are annotated in both species (based on Cliv_2.1; Holt et al., 2018 and Gallus_gallus-5.0; Warren et al., 2017), resulting in comparison of 933/978 pigeon and 1667/2160 chicken genes. Despite this limitation, we found 370 genes that are differentially expressed between FL and HL in both chicken and pigeon (intersection p-value = 3.1e-61; Fig. 7C and Supplemental Table 6). In both species, *TBX5* stood out as the most FL-enriched gene, while TFs *HOXD8, HOXD9, HOXD10*, and *ALX1* were also highly FL-enriched (Log2FC > 2) in both species (Fig. 7D and E). A small set of TFs (*TBX4, PITX1, HOXB9, HOXC10, HOXC11, ISL1*) was highly HL-enriched (Log2FC > 2) in both species (Fig. 7D,F). In both chickens and pigeons, many other TFs were expressed in a FL- or HL-specific manner to a lesser degree (Fig. 7D–F and Supplemental Table 6). Notably, 20% (76/370) of the genes identified in both the pigeon and chicken FL vs. HL datasets were TFs (Supplemental Table 6), further suggesting that TFs are highly conserved components of limb identity programs.

2.9. Evidence for conservation of limb identity programs among amniotes

Recent studies have characterized embryonic FL and HL bud transcriptomes in several mammalian species, including mouse, opossum, pig, and bat (Taher et al., 2011; Maier et al., 2017; Sears et al., 2018; Wang et al., 2018; Gyurjan et al., 2011; Eckalbar et al., 2016; Shou et al., 2005; Nemec et al., 2017). However, similar analyses in other vertebrate classes are sparse, precluding broader comparative analyses of limb identity networks. We therefore extended our analyses by identifying differentially expressed genes in embryonic FLs and HLs of brown anole lizards (*Anolis sagrei*, Supplemental Fig. 14). In addition, we re-analyzed several publicly available mammalian (mouse, opossum, and bat) limb bud datasets (Nemec et al., 2017; Maier et al., 2017; Amandio et al., 2016; Eckalbar et al., 2016). Together with our pigeon and chicken datasets, these additional data enabled us to identify conserved components of the limb identity network in Aves (pigeon and chicken), Sauropsida (pigeon, chicken, and anole), Mammalia (mouse, opossum, and bat), and Amniota (pigeon, chicken, anole, mouse, opossum, and bat) (Fig. 8A). To facilitate comparisons among species, we categorized samples from each dataset into ridge, bud, or paddle developmental stages, which approximately correspond to chicken stages HH21, HH25, and HH29, respectively.

Similar to the avian PCA results, limb buds from anole, mouse, opossum, and bat datasets generally clustered based on limb type (FL vs. HL) and stage (for datasets that included multiple stages) (Fig. 8B–F). For each dataset, we identified genes that are differentially expressed between stage-matched FL and HL buds (Supplemental Table 7). Consistent with our results from pigeon and chicken, in most data sets, almost all gene expression changes between FL and HL are low-level (Log2FC < 1), with only a small set of highly differentially expressed genes (Log2FC > 2) (Supplemental Fig. 15).

To identify deeply conserved limb identity genes, we compared the differentially expressed gene sets from all six amniote species. Despite caveats associated with this type of meta-analysis that undoubtedly reduce our power to detect conserved genes (addressed in Discussion), we identified differentially expressed genes that are associated with FL/HL limb identity in Mammalia (1489 in mouse, opossum, and/or bat), Sauropsidia (56 in anole and pigeon or chicken), and Amniota (44 in anole, pigeon or chicken, and mouse, opossum, or bat) (Fig. 8G and Supplemental Table 7). Within both the Sauropsidia and Amniota gene sets, ~50% (25/56 in Sauropsidia, 21/44 in Amniota) of the conserved differentially expressed genes are TFs (Supplemental Table 7), again highlighting that TFs are a highly conserved component of limb identity networks.

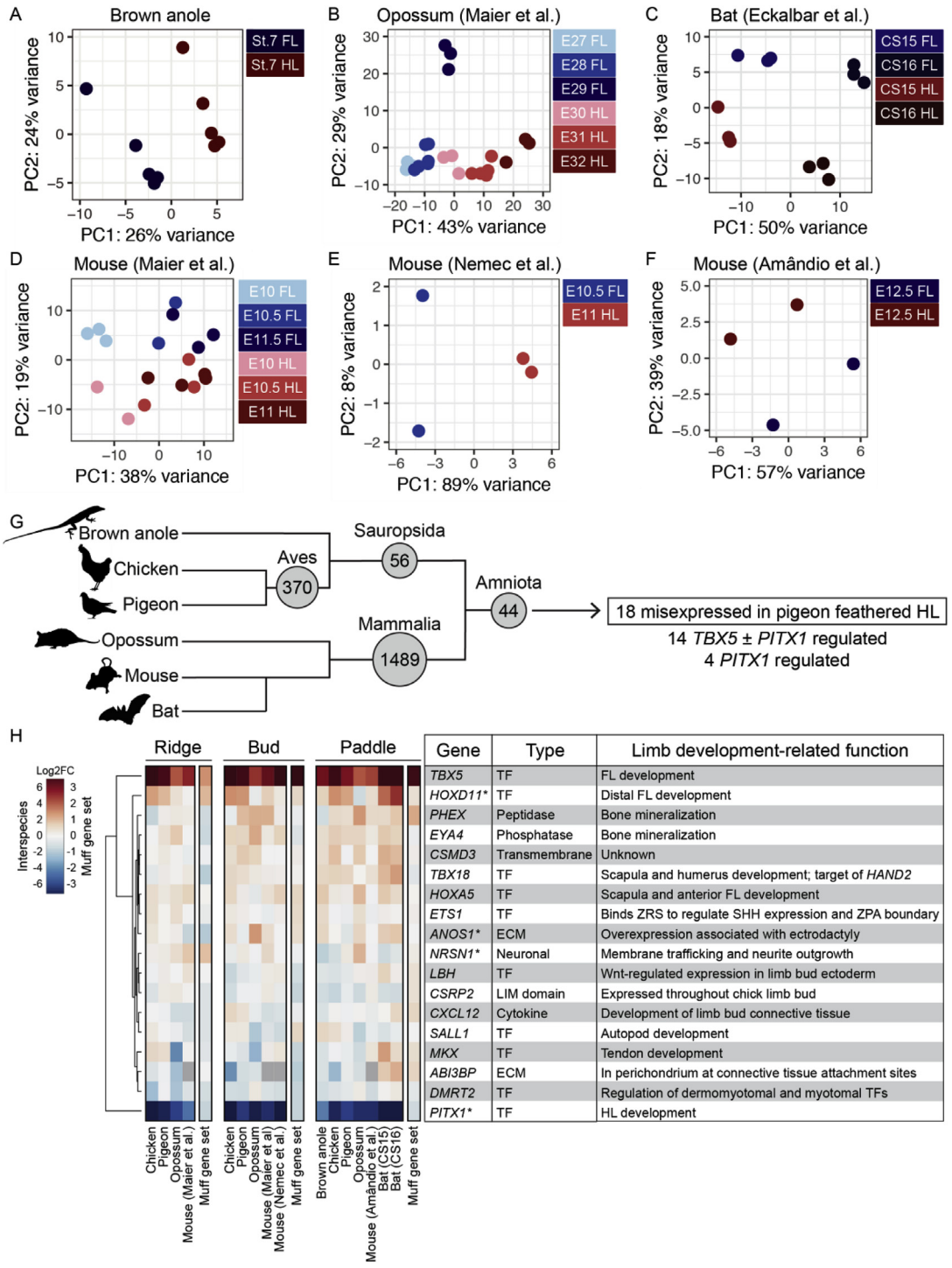
Finally, we asked if the genetic program associated with feathered HL development in pigeons represents a co-option of deeply conserved components of the FL identity program. We compared the grouse and muff gene sets to the 44 limb identity genes conserved in Amniota and found that 41% (18/44) of deeply conserved limb identity genes are also differentially expressed in grouse and/or muff pigeon HLs (Fig. 8G and H). In addition to *PITX1*, we identified 3 deeply conserved limb identity genes (*HOXD11*, *ANOS1*, *NRSN1*) that are misexpressed in both grouse and muff HLs, suggesting that these genes are conserved downstream targets of *PITX1* (Fig. 8H). In addition to *TBX5*, 13 deeply conserved limb identity genes *(PHEX, EYA4, CSMD3, TBX18, HOXA5, ETS1, LBH, CSRP2, CXCL12, SALL1, MKX, ABI3BP, DMRT2)* are misexpressed only in muff HLs (Fig. 8H). This set of 13 genes, which includes 7 TFs, is putatively regulated by *TBX5* or co-regulated by *TBX5* and *PITX1*. In particular, *CXCL12*, *MKX*, and *DMRT2* may be co-regulated by *TBX5* and *PITX1*, as these genes were recently identified as conserved regulatory targets of *PITX1* in mouse and anole HLs (Wang et al., 2018). Of the 18 deeply conserved limb identity genes that are deployed during pigeon feathered HL development, most have been implicated in specific aspects of limb development with varying degrees of supporting evidence (*TBX5*, *HOXD11*, *TBX18*, *HOXA5*, *ETS1*, *LBH*, *CSRP2*, *SALL1*, *CXCL12*, *MKX*, *PITX1*) (Boulet and Capecchi, 2004; Zakany and Duboule, 2007; Sheeba and Logan, 2017; Haraguchi et al., 2015; Xu et al., 2013; Lettice et al., 2012; Kvon et al., 2016; Briegel and Joyner, 2001; Bonnin et al., 2002; Kawakami et al., 2009; Farrell and Munsterberg, 2000; Nassari et al., 2017; Odemis et al., 2005; Liu et al., 2010; Ito et al., 2010; Duboc and Logan, 2011b) (Fig. 8H). For others (*EYA4*, *ANOS1*, *PHEX*, *NRSN1*, *DMRT2*, *ABI3BP, CSMD3*), a link to limb development has not been established. Furthermore, for most of the 18 deeply conserved limb identity genes, the precise regulatory connections to *TBX5* and/or *PITX1* remain open for discovery.

3. Discussion

We identified transcriptional changes associated with differences in FL, HL, and limbs with mixed identities. We took advantage of regulatory variants associated with pigeon foot feathering to specifically determine how shifts in the expression of two regulators of limb identity, *PITX1* and *TBX5*, affect transcription of downstream genes during embryonic limb development. We found that most gene expression differences between FLs and HLs are subtle, and that only a small set of genes is highly differentially expressed. We identified suites of genes encoding TFs, ECM proteins, and components of developmental signaling pathways with known roles in limb development that are regulated downstream of *TBX5* and/or *PITX1*. Finally, by comparing limb identity networks across amniotes, we found a small group of deeply conserved gene expression changes associated with limb identity. Some of the genes in this core group are also misexpressed in feathered pigeon HLs, consistent with a partial HL to FL transformation at both the phenotypic and transcriptomic level.

3.1. Embryonic limb identity networks

In birds, we found that limb type-specific expression of a small set of TFs is coupled with an accumulation of widespread low-level gene expression changes to form radically divergent FL and HL morphologies. Notably, transcriptomic studies of mouse embryonic limb development identified a similar number and level (i.e. fold-change) of gene expression changes between FL and HL (Nemec et al., 2017). These parallel



Gene	Type	Limb development-related function
TBX5	TF	FL development
*HOXD11**	TF	Distal FL development
PHEX	Peptidase	Bone mineralization
EYA4	Phosphatase	Bone mineralization
CSMD3	Transmembrane	Unknown
TBX18	TF	Scapula and humerus development; target of *HAND2*
HOXA5	TF	Scapula and anterior FL development
ETS1	TF	Binds ZRS to regulate SHH expression and ZPA boundary
*ANOS1**	ECM	Overexpression associated with ectrodactyly
*NRSN1**	Neuronal	Membrane trafficking and neurite outgrowth
LBH	TF	Wnt-regulated expression in limb bud ectoderm
CSRP2	LIM domain	Expressed throughout chick limb bud
CXCL12	Cytokine	Development of limb bud connective tissue
SALL1	TF	Autopod development
MKX	TF	Tendon development
ABI3BP	ECM	In perichondrium at connective tissue attachment sites
DMRT2	TF	Regulation of dermomyotomal and myotomal TFs
*PITX1**	TF	HL development

Fig. 8. Identification of deeply conserved elements of limb identity program in amniotes. (**A-F**) PCAs based on top 500 most variable genes from brown anole (A), opossum (B), bat (C), and three different mouse (D–F) limb bud datasets. (B) and (D) are analyses of datasets from (Maier et al., 2017), (C) is analysis of dataset from (Eckalbar et al., 2016), (E) is analysis of dataset from (Nemec et al., 2017), and (F) is analysis of dataset from (Amandio et al., 2016). For (A–F), specific stages of samples are listed and color coded based on stage group. (**G**) Tree summarizing evolutionary relationships between species included in comparative transcriptomics analyses. Number of genes differentially expressed in FL vs. HL of Aves (chicken and pigeon), Mammalia (opossum, bat, and/or mouse), Sauropsida (brown anole and chicken or pigeon), and Amniota (brown anole, chicken or pigeon, and opossum, bat, or mouse) are denoted in gray circles on the tree. Of the 44 genes differentially expressed in FL vs. HL across amniotes, 18 are also misexpressed in pigeon feathered HLs. Of these, 4 genes are putative *PITX1* targets (misexpressed in grouse and muff HLs relative to scaled HLs) and 14 are likely regulated by *TBX5* or co-regulated by *TBX5* and *PITX1* (misexpressed in muff HLs, but not grouse HLs). (**H**) Gene-wise hierarchical clustering heat map of 18 genes that are differentially expressed in FL vs. HL across amniotes and scaled vs. feathered HL in pigeons. The scale for between-species comparisons is Log2FC −6 (HL-enriched) to 6 (FL-enriched); the scale for pigeon HL comparisons is Log2FC −3 (scaled HL-enriched) to 3 (muff HL-enriched). Gray boxes within the heat map denote no data (*ABI3BP* is not annotated in mouse genome). Table summarizes 18 deeply conserved genes, including type of gene and limb development-related function (if known). Asterisks (*) denote 4 putative *PITX1* targets. Credits for animal silhouettes in G: Sarah Werning (anole and opossum), Steven Traver (chicken), Dori and Nevit Dilment (pigeon), David Liao (mouse), and Yan Wong (bat).

findings are particularly interesting considering that variation in adult limb morphologies of mice (FL and HL are covered with hair and have similar skeletal proportions) is less dramatic than in birds (FLs are feather covered wings, HLs are scaled covered legs, and limb skeletons are highly divergent). Thus, the identity of the differentially expressed genes, and not the overall number or level of gene expression changes, ultimately determines adult limb phenotype. In our most phylogenetically inclusive analyses, we identified a core set of gene expression changes associated with amniote FL/HL limb identity, but we also found many genes that are differentially expressed between FLs and HLs in a species-specific manner. We predict that these unique sets of genes are associated with the development of species-specific limb phenotypes. In support of this idea, specific sets of genes initiate distinct developmental programs to form feathers or different scale types in archosaur embryos (Musser et al., 2018; Wu et al., 2018; Lai et al., 2018).

3.2. Parsing TBX5 vs. PITX1-dependent regulation of the limb identity network

Pigeon foot feathering offers a unique opportunity to understand the independent and combinatorial effects of *PITX1* and *TBX5* expression changes on limb patterning. Both grouse and muff HLs are feathered, but the combination of reduced *PITX1* and ectopic *TBX5* expression (muff) has a more pronounced effect on the limb regulatory network than reduced *PITX1* expression alone (grouse). Because our comparative analyses were performed using samples from one scaled breed (Racing Homer), one grouse breed (Oriental Frill), and one muff breed (English Trumpeter), it is possible that some of the differentially expressed genes we detected between scaled and feathered HLs represent breed-specific shifts in gene expression that are independent of the HL appendage phenotype. However, by comparing grouse and muff gene sets, we were able to infer a set of 601 putative *PITX1*-specific transcriptional targets that are differentially expressed in both grouse and muff HLs relative to scaled HLs. Validating specific genes of interest as direct downstream targets of *PITX1* remains an important avenue of future research. In the present study, we were unable to separate *TBX5*-specific from *TBX5/PITX1*-synergistic effects in pigeon limb development. This shortcoming could be resolved by analyzing HL bud transcriptomes from feather-footed pigeons that carry the *Slipper* (*TBX5*) allele but not the *grouse* (*PITX1*) allele, although such breeds are rare (English and Pigmy Pouters only).

3.3. Partial co-option of pigeon FL identity program in feathered HLs

By comparing the differentially expressed genes from scaled and feather-footed pigeons, we found that the gene expression programs controlling feathered HL development are partially co-opted from the normal FL program. Thus, in feather-footed pigeons, a portion of the FL program is redeployed in the HL to form feathered HLs with partial anatomical and molecular FL identity. Spatiotemporal modulation of gene expression, particularly of genes like *PITX1* and *TBX5* that function at the top of gene regulatory hierarchies, is an important driver of evolutionary diversification (Davidson, 2006; Erwin and Davidson, 2009; Rebeiz et al., 2015; Infante et al., 2018). Generation of phenotypic diversity and novelty via co-option of genetic circuits or top-level network regulators is a repeated theme in evolution; notable examples include co-option of the adult skeletogenic network to form the larval skeleton in echinoderms, redeployment of the highly conserved arthropod appendage-patterning network to form beetle horns, and overlap of the genetic networks that control embryonic limb and external genital development in tetrapods (Rebeiz et al., 2015; Davidson, 2006; Erwin and Davidson, 2009; Infante et al., 2015, 2018; Gao and Davidson, 2008; Tschopp et al., 2014; Herrera and Cohn, 2014; Leal and Cohn, 2016, 2018). Our comparative analyses in pigeons indicate that TFs are a major component of the FL identity program redeployed in feathered HLs; similarly, others have noted that genes at the top of regulatory networks are more evolutionarily stable than differentiation genes at the network periphery (Davidson, 2006; Erwin and Davidson, 2009; Rebeiz et al., 2015). Within the co-opted FL program in feathered HLs, we also identified genes encoding developmental signaling pathway components and ECM proteins, which link subcircuits of the regulatory network and function as differentiation genes at the network periphery, respectively (Erwin and Davidson, 2009; Davidson, 2006; Rebeiz et al., 2015). These findings suggest that changes in the expression of top-level regulators (*PITX1* and/or *TBX5*) have widespread effects on all levels of the limb development network.

Despite a partial co-option of the FL identity program, it is important to note that pigeon feathered HLs are not FLs. They are not the consequence of a complete redeployment of the FL identity network onto a blank canvas. Instead, the ectopic FL program is superimposed onto the resident HL regulatory network, resulting in a HL with mixed identity. During normal limb bud development, ectodermal identity is determined in response to signals from the underlying mesoderm (Cairns and Saunders, 1954; Saunders et al., 1959; Sengel, 1990; Hughes et al., 2011). In muff HLs, ectopic and spatially-restricted mesodermal expression of *TBX5* signals to the ectoderm to form feathered epidermis. The largest flight-like feathers of muffed pigeon feet develop superficial to the ectopic *TBX5* expression domain in the posterior-dorsal limb bud. This spatially-restricted FL-like genetic program occurs within the context of a HL identity program with reduced *PITX1* expression. Together, changes in *PITX1* and *TBX5* expression cause a reorganization of the downstream limb identity network that results in a limb that is neither completely HL nor completely FL, as is evident from the changes in both epidermal appendage type and musculoskeletal patterning (Domyan et al., 2016). This layering of multiple genetic networks represents an intriguing potential mechanism of phenotypic diversification.

3.4. Deeply conserved components of limb identity networks

By comparing FL and HL bud transcriptomes from several amniote species, we identified a core set of deeply conserved limb identity genes that is enriched for genes encoding TFs, ECM proteins, and signaling pathway components. We suspect that only a subset of the deeply conserved genes was identified in our analyses, due primarily to technical caveats commonly associated with genomic analyses (e.g., differences in sample preparation, sequencing method, quality of reference genome assembly and annotation). For example, in our analyses of brown anole (*Anolis sagrei*) limb buds, RNA-seq reads had to be aligned to the genome of the closely related green anole (*Anolis carolinensis*) because a brown anole genome assembly is not currently available. Analysis modifications like this could reduce our ability to detect differentially expressed genes. Nevertheless, our analyses suggest that the number of genes differentially expressed between FL and HL in a species-specific manner is considerably greater than the number of deeply conserved limb identity genes. This result suggests that relatively few genes are absolutely required for amniote FL vs. HL identity and raises the possibility that there is considerable evolutionary variation in the genes that were recruited to the limb development and identity programs. Another classic example of this paradigm is animal sex determination, in which a small set of master sex determination genes are highly conserved across species (e.g. *DMRT1*, *SOX3*), but the genetic players vary dramatically at the network periphery (Herpin and Schartl, 2015).

Of the deeply conserved FL/HL identity genes we identified, a subset was also differentially expressed between scaled and feathered pigeon HLs, suggesting that the genetic program underlying pigeon foot feathering is not completely novel, but instead incorporates elements of the deeply conserved FL/HL limb identity network. In the future, it will be exciting to determine if similar mechanisms of network co-option and/or overlaying of multiple regulatory networks are broadly associated with evolutionary diversification of vertebrate limbs and other structures.

4. Materials and methods

4.1. Animal husbandry, isolation of embryonic tissue and sample sex determination

Columba livia were housed in accordance with the University of Utah Institutional Animal Care and Use Committees of University of Utah (16–03010). Pigeon eggs were collected from Racing Homer (scaled), Oriental Frill (grouse) and English Trumpeter (muff) breeding pairs and incubated to the desired embryonic stage. White Leghorn chicken eggs were obtained from AA Lab Eggs (Westminster, California) and incubated to the desired stage. For avian samples, pairs of forelimb (FL) and hindlimb (HL) buds were dissected from scaled, grouse and muff pigeon embryos or chicken embryos at HH21 (embryonic day E3.5), HH25 (E4.5), and HH29 (E6) and stored in RNAlater (ThermoFisher Scientific) at −80 °C. Additional tissue was harvested from each embryo and stored at −80 °C for DNA extraction and genotyping. Genomic DNA was isolated from each embryonic tissue sample using a DNeasy Blood and Tissue Kit (Qiagen). For each pigeon sample, sex was determined using a previously published PCR-based assay (Fridolfsson and Ellegren, 1999).

Anolis sagrei were maintained at the University of Georgia following published guidelines (Sanger et al., 2008a). Animals were wild individuals captured at the Fairchild Tropical Botanic Gardens in Miami, FL. Breeding cages housed up to 4 adult females and 1 adult male together. Eggs were collected from nest boxes weekly and incubated at 26 °C. Pairs of FL and HL buds were dissected from Stage 7 *A. sagrei* embryos and stored in RNAlater at −80 °C. Embryos were staged according to (Sanger et al., 2008b). All experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were performed with the approval and oversight of the University of Georgia Institutional Animal Care and Use Committee (A2015 02-020-Y3-A13).

4.2. RNA isolation, cDNA synthesis, and qRT-PCR analysis

Total RNA was extracted from FL and HL bud samples using the RNeasy Mini Kit with RNase-Free DNAse Set and a TissueLyser LT (Qiagen). For each sample, a cDNA library was prepared using M-MLV Reverse Transcriptase with oligo(dT) primer (ThermoFisher Scientific). Intron-spanning amplicons from pigeon *PITX1*, *TBX5*, and *ACTB* were amplified from cDNA libraries using a CFX96 instrument and iTaq Universal Sybr Green Supermix (BioRad). Primer sequences were published previously (Domyan et al., 2016). For a single qRT-PCR experiment, three technical replicates were performed for each sample and the mean value was determined. Each experiment was repeated three times. Statistical significance was determined using a pairwise Wilcoxon rank sum test in R version 3.3.3 (R Core Team, 2018).

4.3. Library preparation and RNA sequencing of avian samples

RNA-sequencing libraries were prepared and sequenced by the High-Throughput Genomics and Bioinformatic Analysis Shared Resource at the University of Utah. RNA sample quality was assessed using the RNA ScreenTape Assay (Agilent); RNA integrity (RIN) scores are presented in Supplemental Table 8. For each sample, a sequencing library was prepared using the TruSeq Stranded mRNA Sample Prep Kit with oligo(dT) selection (Illumina). 125-cycle paired-end sequencing was performed on an Illumina HiSeq 2500 instrument (12 libraries/lane). An average of 21 million reads were generated for each pigeon sample and 25 million reads for each chicken sample.

4.4. Analysis of avian RNA-seq data

Sequencing read quality was assessed with FastQC (Babraham Bioinformatics). Read alignment was performed using STAR version 2.5.0a (Dobin et al., 2013). Illumina adapters were trimmed and reads were aligned to the pigeon Cliv_2.1 reference assembly (Holt et al., 2018) or chicken Gallus_gallus-5.0 genome assembly (Warren et al., 2017) with STAR using the 2-pass mode. GTF annotation files were used to guide spliced read alignments. The average percentage of uniquely mapped reads was 84.07% for pigeon samples and 91.45% for chicken samples. Mapped reads were assigned to genes using featureCounts from the Subread package version 1.5.1 (Liao et al., 2014), with an average of 72.36% of reads assigned in pigeon samples and 74.48% in chicken samples. Principal component analyses (PCA) and differential expression analyses were performed with the R package DESeq2 version 1.12.4 (Love et al., 2014). Read count files were imported into DESeq2 and a DESeqDataSet object was generated that included sample sex and sequencing lane as covariates. Prior to running any DESeq2 functions, datasets were pre-filtered to remove genes with a total of 0 reads across all samples. For PCA, regularized log (rlog) transformation (RLT) or variance stabilized transformation (VST) was applied to the entire pigeon or chicken dataset using built-in DESeq2 commands. To generate differentially expressed gene lists, we employed a Benjamini & Hochberg adjusted p-value cutoff of $p < 0.05$ because in some cases, more conservative cutoffs resulted in gene sets that did not include *PITX1* or other known limb identity factors in the FL vs. scaled HL comparisons. The R package GeneOverlap version 1.18.0 (Shen and Sinai, 2018) was used with default settings to test for statistically significant intersections between sets of differentially expressed genes. Background genome size was set to 15392 (pigeon vs. pigeon comparisons), 22604 (mouse vs. mouse comparison), or 11202 (pigeon vs. mouse comparisons). In order to perform downstream comparative analyses, differentially expressed gene lists were annotated with human gene names using a custom R script (available as a Supplemental File).

4.5. Analysis of transcription factors, extracellular matrix components, and signaling pathways.

To identify transcription factors (TFs) within our differentially expressed gene lists, we cross-referenced gene lists to a list of ~1400 genome-wide TFs compiled from AnimalTFDB 2.0 (Zhang et al., 2015). Extracellular matrix genes were identified by comparing differential expression results to a list of genes associated with the GO term "extracellular matrix" (GO:0031012). Ingenuity Pathway Analysis 2.2 (IPA, Qiagen) was used to identify differentially expressed components of specific developmental signaling pathways with roles in limb development. We specifically searched for significant enrichment of the following canonical pathways in IPA: Wnt signaling (Wnt/β-catenin signaling, PCP pathway, Wnt/Ca + signaling), BMP signaling, TGF-β signaling, Sonic Hedgehog signaling, and RAR activation.

4.6. RNA-seq and analysis of brown anole (Anolis sagrei) limb buds

A. sagrei FL and HL RNA-seq libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit with oligo(dT) selection (Illumina). Five separate FL and HL library sets were prepared from limb RNA collected from five different embryos. 75-cycle paired-end sequencing was performed on an Illumina NextSeq 500 instrument (10 libraries/lane). An average of 43 million reads were generated for each *A. sagrei* library. Read quality assessment, adapter trimming, and mapping to the Green anole (*Anolis carolinensis*) AnoCar2.0 genome assembly were performed as described above with modified alignment parameters (–outFilterScoreMinOverLread 0.3 –outFilterMatchNminOverLread 0.3 –outFilterMultimapNmax 15) to allow for mismatches due to species-specific sequence differences. The average percentage of uniquely mapped reads was 79.19% for brown anole samples. Mapped reads were assigned as described above, with an average of 61.23% of reads assigned. PCA was performed using the top 100 most differentially expressed genes and FL vs. HL differential expression analyses were performed using DESeq2 as described above.

4.7. Analysis of publicly available mammalian RNA-seq datasets and comparative transcriptomics between species

Raw RNA-seq reads from three limb bud datasets from mouse (*Mus musculus*; Maier et al., 2017; Nemec et al., 2017; Amandio et al., 2016), one limb bud dataset from opossum (*Monodelphis domestica*; Maier et al., 2017), and one limb bud dataset from bat (*Miniopterus natalensis*; Eckalbar et al., 2016) were downloaded from the GEO (GSE71390, GSE79028, GSE100734) and BioProject (PRJNA270639) databases. Read quality assessment, adapter trimming, and read mapping to mouse GRCm38.p6, opossum monDom5, or bat Mnat.v1 genome assemblies were performed as described above, with an average alignment percentage of 86.90% (mouse; Maier et al., 2017), 76.46% (mouse; Nemec et al., 2017), 88.43% (mouse; Amandio et al., 2016), 84.76% (opossum; Maier et al., 2017), and 92.53% (bat; Eckalbar et al., 2016). Aligned reads were assigned to genes as described above, with an average of 66.47% (mouse; Maier et al., 2017), 48.80% (mouse; Nemec et al., 2017), 70.90% (mouse; Amandio et al., 2016), 58.93% (opossum; Maier et al., 2017), and 50.40% (bat; Eckalbar et al., 2016) successfully assigned reads. For each dataset, PCA and FL vs. HL differential expression analyses were performed using DESeq2 as described above. A master list of differentially expressed genes in mouse limb buds was generated by combining the gene sets obtained from the mouse datasets.

To facilitate comparative transcriptomic analyses, gene lists from all species were annotated with human gene names using a custom R script. To identify differentially expressed genes conserved in Aves, we intersected FL vs. HL gene lists from pigeon and chicken. To find genes conserved in Mammalia, we intersected the mouse, opossum, and bat gene sets and identified genes differentially expressed in at least two species. FL vs. HL genes conserved in Sauropsida were determined by intersecting gene lists from pigeon, chicken, and brown anole and identifying genes that are differentially expressed in brown anole and at least one member of Aves (pigeon and/or chicken). Similarly, FL vs. HL genes conserved in Amniota were determined by identifying genes that are differentially expressed in brown anole, at least one member of Aves (pigeon and/or chicken) and at least one member of Mammalia (mouse, opossum, and/or bat). Finally, we cross-referenced the Amniota gene list to the pigeon scaled HL vs. grouse HL and scaled HL vs. muff HL gene lists to identify deeply conserved FL vs. HL genes that are also misexpressed during grouse and/or muff HL development.

4.8. Photo and image credits

Photos used in Fig. 1 were taken by Sydney Stringham (A, Racing Homer) and Thomas Hellmann (B, Oriental Frill and C, English Trumpeter). Animal silhouettes used in Fig. 8G were downloaded as vector images from PhyloPic (phylopic.org). The silhouettes were created by Sarah Werning (anolis and opossum), Steven Traver (chicken), Dori and Nevit Dilmen (pigeon), David Liao (mouse), and Yan Wong (bat) and reused under the Creative Commons Attribution-ShareAlike 3.0 Unported license (mouse and pigeon; https://creativecommons.org/licenses/by-sa/3.0/), the Creative Commons Attribution 3.0 Unported license (anolis and opossum; https://creativecommons.org/licenses/by/3.0/), or the Public Domain Dedication 1.0 license (chicken and bat).

Competing interests

No competing interests declared.

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Data availability

All RNA-seq data generated for this study have been deposited at Gene Expression Omnibus under accession numbers GSE127775 (*C. livia* and *G. gallus*) and GSE128151 (*A. sagrei*).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.06.015.

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