Genetic Interactions Between *Shox2* and *Hox* Genes During the Regional Growth and Development of the Mouse Limb

Stanley J. Neufeld,* Fan Wang,† and John Cobb*,[1]

*Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada, and †Department of Neurobiology, Duke University Medical Center, Durham, North Carolina 27710

ABSTRACT The growth and development of the vertebrate limb relies on homeobox genes of the *Hox* and *Shox* families, with their independent mutation often giving dose-dependent effects. Here we investigate whether *Shox2* and *Hox* genes function together during mouse limb development by modulating their relative dosage and examining the limb for nonadditive effects on growth. Using double mRNA fluorescence *in situ* hybridization (FISH) in single embryos, we first show that *Shox2* and *Hox* genes have associated spatial expression dynamics, with *Shox2* expression restricted to the proximal limb along with *Hoxd9* and *Hoxa11* expression, juxtaposing the distal expression of *Hoxa13* and *Hoxd13*. By generating mice with all possible dosage combinations of mutant *Shox2* alleles and *HoxA/D* cluster deletions, we then show that their coordinated proximal limb expression is critical to generate normally proportioned limb segments. These epistatic interactions tune limb length, where *Shox2* underexpression enhances, and *Shox2* overexpression suppresses, *Hox*-mutant phenotypes. Disruption of either *Shox2* or *Hox* genes leads to a similar reduction in *Runx2* expression in the developing humerus, suggesting their concerted action drives cartilage maturation during normal development. While we furthermore provide evidence that *Hox* gene function influences *Shox2* expression, this regulation is limited in extent and is unlikely on its own to be a major explanation for their genetic interaction. Given the similar effect of human *SHOX* mutations on regional limb growth, *Shox* and *Hox* genes may generally function as genetic interaction partners during the growth and development of the proximal vertebrate limb.

THE vertebrate limb is a valuable model for studying the genetic coordination of a complex developing structure. The proximodistal axis of the limb is composed of discrete segments, the growth and development of which are selectively perturbed when individual, or combinations of, homeobox genes are disrupted. In mice, mutations of the paralogous *Hox9* and *Hox10* genes result in shortened stylopodal elements (containing the humerus and femur) (Fromental-Ramain *et al.* 1996a; Wellik and Capecchi 2003), deletions of *Hox11* genes result in truncated zeugopodal elements (radius/ulna and fibula/tibia) (Davis *et al.* 1995; Wellik and Capecchi 2003), and disruption of *Hox13* genes results in agenesis of the autopod (metacarpals/metatarsals and the digits) (Fromental-Ramain *et al.* 1996b). Mutation of *short stature homeobox* (*Shox*) genes similarly gives rise to the disproportionate shortening of certain limb regions. In humans, loss of *SHOX* leads to the truncated zeugopod elements found in people with Leri–Weill, Turner, and Langer syndromes (Rao *et al.* 1997; Belin *et al.* 1998; Shears *et al.* 1998; Zinn *et al.* 2002). While rodents have uniquely lost the *Shox* gene among mammals (Gianfrancesco *et al.* 2001), disruption of the widely conserved *Shox2* gene results in severely shortened stylopodal elements in mice (Cobb *et al.* 2006). Thus, *Hox* and *Shox* gene perturbations each give rise to regional phenotypes along the proximodistal axis, suggesting the possibility that these genes function together during limb development.

Limb chondrogenesis begins following the early stages of limb bud formation, where mesenchymal cells condense and differentiate into *Col2a1*-expressing chondrocytes. After a proliferative phase, the chondrocytes nearest the middle of the element stop dividing, undergo hypertrophy, and express *Col10a1*, a process that is associated with elongation of the skeletal element (Karsenty and Wagner 2002). Surrounding the chondrocytes is a layer of flattened and elongated cells, the perichondrium, that influences the developmental

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[1]Corresponding author: 2500 University Dr. NW, University of Calgary, Calgary, AB T2N 1N4, Canada. E-mail: jacobb@ucalgary.ca

progression of the cartilage cells and is furthermore important for growth (Kronenberg 2007). *Runx2*, which is expressed both by chondrocytes and the perichondrium, is essential for proper chondrocyte hypertrophy and the formation of osteoblasts, thus being important for both the proper development of the cartilage template and its eventual replacement by bone (Otto *et al.* 1997; Yoshida *et al.* 2004). As mutation of *Shox2* or *Hox* genes results in a strong reduction or loss of *Runx2* expression, a lack of chondrocyte maturation likely underlies regional shortening in these animals (Boulet and Capecchi 2004; Cobb *et al.* 2006; Villavicencio-Lorini *et al.* 2010; Gross *et al.* 2012).

The regulation of individual *Hox* and *Shox* genes follows precise spatial and temporal patterns of gene expression in the limb. The regulation of *Hox* genes has been intensively studied, showing that their expression occurs in two main phases: an early phase of gene expression that is associated with the development of the more proximal limb (stylopod and zeugopod) and a later phase that is associated with the development of the distal limb (autopod) (Kmita *et al.* 2002; Tarchini and Duboule 2006; Andrey *et al.* 2013). This collinear strategy sets up partially overlapping domains of gene expression across the limb that underlie the discrete phenotypes that occur when paralagous *Hox* genes are mutated. The reported expression of human *SHOX* and *SHOX2* is also regional, with *SHOX2* expressed in the developing stylopod and *SHOX* expressed in the developing zeugopod (Clement-Jones *et al.* 2000). Mouse *Shox2* expression, in contrast, occupies both the developing stylopodal and zeugopodal domains even though its mutation primarily disrupts the developing stylopod (Cobb *et al.* 2006). Within their broad segmental domains, *Shox2* and at least some *Hox* genes are expressed in the proliferating chondrocytes and perichondrium of developing skeletal elements (Villavicencio-Lorini *et al.* 2010; Swinehart *et al.* 2013), being thus associated with their continual growth during development.

Gene expression levels can be critical during development, with variation in expression leading to dose-dependent responses. The effects of dosage variation can be assessed by modulating the expression of a single gene or varying the expression of multiple genes, where genetic interactions, or epistasis, become important. In the latter case, the effect of a given variant differs in the presence and absence of variation in another gene (Phillips 2008). For *Hox* genes, which harbor a large degree of functional redundancy, the quantitative nature of their function is manifest when a certain threshold of gene product is crossed. For instance, removing a single allele of *Hoxa13* in an otherwise wild-type animal has far less effect on development than when it is removed in conjunction with *Hoxd13* disruption (Fromental-Ramain *et al.* 1996b). The quantitative response to *Hox* gene function has been proposed to distinguish "short" bones from "long" bones (Gonzalez-Martin *et al.* 2014). *SHOX* function in humans is also dosage sensitive; a majority of individuals missing one allele of *SHOX* have moderately shortened zeugopodal segments, while individuals missing both *SHOX* alleles have a more penetrant and severe shortening (Rao *et al.* 1997; Belin *et al.* 1998; Shears *et al.* 1998; Zinn *et al.* 2002; Albuisson *et al.* 2012). *SHOX* duplications have furthermore been hypothesized to lead to tall stature (Ogata *et al.* 2000; Durand and Rappold 2013), highlighting the interest in *SHOX* dosage and its effect on limb development. Thus, examining the effects of dosage variation is key to understanding the role of *Shox* and *Hox* genes in development and disease.

In the present work, we investigate the functional relationship between *Shox2* and *Hox* genes during mouse limb development. Modulating *Shox2* transcript levels in a variety of *Hox*-mutant backgrounds produces nonadditive effects on limb growth, indicative of genetic interaction. This approach reveals that the functions of these genes are intimately associated, exhibiting aspects of both synergy and redundancy during limb development and functioning upstream of *Runx2* during chondrogenesis.

Materials and Methods

Mice

Mouse lines used were $Shox2^{fl/+}$ (Cobb *et al.* 2006), $Rosa^{CAG\text{-}STOP\text{-}Shox2}$ (Scott *et al.* 2011), $HoxD^{+/-}$ *(Del9)* (Spitz *et al.* 2001), $HoxA^{fl/+}$ (Kmita *et al.* 2005), and *Prrx1-Cre* (Logan *et al.* 2002). The Life and Environmental Sciences Animal Care Committee approved all animal experiments.

In situ hybridization

Chromogenic or fluorescence whole-mount *in situ* hybridization was performed as previously outlined (Neufeld *et al.* 2013). For confocal microscopy, limb buds were imaged in 1% low-melt agarose. Single optical sections were taken at 40×, using a Zeiss LSM-510 META confocal microscope. Section ISH was performed on 10-μm cryosections as previously described (Alam *et al.* 2005) except that BMpurple (Roche) was used for signal development. Probes used were *Shox2* (Cobb and Duboule 2005), *Hoxd9* (Renucci *et al.* 1992), *Hoxa11* (Cobb and Duboule 2005), *Hoxd13* (Dolle *et al.* 1991), *Hoxa13* (Warot *et al.* 1997), *Col2a1* (Metsaranta *et al.* 1991), *Runx2* (Cobb *et al.* 2006), and *Col10a1* (Apte *et al.* 1992).

Quantitative real-time PCR

Embryos were dissected in PBS and the limb buds stored in RNAlater (QIAGEN, Valencia, CA) during genotyping. Limb bud tissue was disrupted using a motorized pestle (VWR), and RNA was isolated using the E.Z.N.A Total RNA Kit I (Omega Bio-Tek, Norcross, GA). RNA was reverse transcribed using qScript cDNA Super Mix (Quanta BioSciences, Gaithersburg, MD). Relative levels of selected cDNAs were measured using PerfeCTa SYBR Green Fastmix (Quanta BioSciences) and an iCycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) by comparison to a standard curve. Levels were normalized to *Actb*. Primer sequences used were *Shox2* forward, TGGAACAACTCAACGAGCTGGAGA; *Shox2* reverse, TTCAAACTGGCTAGCGGCTCCTAT; *Actb*

forward, TTAATTTCTGAATGGCCCAGGTCT; and *Actb* reverse, ATTGGTCTCAAGTCAGTGTACAGG. Differences in transcript levels between groups were assessed with two-tailed unpaired *t*-tests, using between three and five animals per genotype.

Skeletal staining and length quantification

Skeletons of newborn mice were stained with Alizarin Red and Alcian Blue, using standard techniques. The lengths of the Alizarin-Red stained regions of the right stylopodal and zeugopodal elements were measured under a microscope, using a densely marked straightedge, giving measurements to the closest tenth of a millimeter. No differences in overall body length were observed between wild-type animals and mutants.

Graphing

Bar plots and interaction plots were made using GraphPad Prism software. Heat maps in Figure 2 were made using the gplots package in R (R Core Team 2013).

Statistical analysis of genetic Interactions

To identify epistatic interactions between *Shox2* and *Hox* genes during limb growth, the limbs from three to six animals were measured for each of the 36 genotypes analyzed. A factorial analysis of variance (ANOVA) was performed using the lm function in R, considering *Shox2*, *HoxA*, and *HoxD* as independent factors and the number of functional alleles as levels (or groups in each factor). The *RosaShox2* transgene was considered as an additional *Shox2* level. To minimize the number of limb measurements with zero length, which also had zero variance, the $Shox2^{c/-}$ genotype was excluded from the femur data set, and the $HoxD^{-/-}$ genotype was excluded from the radius and ulna data sets. Following these exclusions, the data sets for each element showed roughly uniform variance and nearly normally distributed residuals (as judged by residuals-*vs.*-fitted plots and quantile-quantile). Differential effects of a given *Shox2* mutation between wild-type and mutant *Hox* backgrounds were assessed by performing contrasts, using the multcomp package (Hothorn *et al.* 2008) in R. The estimates from these contrasts can be calculated with the following equation (as seen in Jaccard 1998), substituting in the average lengths (designated μ) of the four following genotypes,

$$\varepsilon = \left(\mu_{\mathrm{Shox2(WT);Hox(WT)}} - \mu_{\mathrm{Shox2(mut);Hox(WT)}}\right) - \left(\mu_{\mathrm{Shox2(WT);Hox(mut)}} - \mu_{\mathrm{Shox2(mut);Hox(mut)}}\right),$$

where ε is the difference between the expected and observed lengths, and Shox2(mut) and Hox(mut) are mutant genotypes for *Shox2* and *Hox* genes. When there is no genetic interaction, the estimate is zero or close to zero. When a genetic interaction is present, the value is significantly different from zero. To account for multiple comparisons, a Bonferroni correction was used to generate a stringent significance threshold.

Results

To investigate the possibility that *Shox2* and *Hox* genes function together during limb development, we examined their relative expression dynamics, using dual fluorescence *in situ* hybridization (FISH) in single embryos (Figure 1). *Hoxd9*, *Hoxa11*, and *Hoxd13* were used as representative *Hox* genes that function in the individual segments along the proximodistal axis. At embryonic day 10.5 (E10.5), *Shox2* transcripts occupied the majority of the limb bud, giving extensive overlap with each *Hox* gene (Figure 1A, inset shows expression of *Shox2* and *Hoxd13* in a single optical section). At E11.5, *Shox2* expression was confined to the proximal limb and shared a similar distal expression border to *Hoxd9* and *Hoxa11* (Figure 1B), the latter of which selectively marks the developing zeugopod (Nelson *et al.* 1996). *Hoxd13* was, in contrast, no longer expressed with *Shox2*, but expanded in the distal region of the limb bud in association with the developing autopod (Figure 1B). At E12.5, *Shox2* expression is maintained proximally, along with *Hoxd9* and *Hoxa11*, and *Hoxd13* expression surrounds the developing digit condensations. As *Hoxa13* is additionally expressed in the developing carpal region (Nelson *et al.* 1996), its domain closely juxtaposes that of *Shox2* (Figure 1C). Thus *Shox2* and *Hox* genes are coordinately expressed in broad domains that closely correspond to the discrete segments of the proximodistal axis. To investigate their expression in and around the proximal skeletal elements, we performed chromogenic ISH on sections at E12.5 and E13.5. *Shox2*, *Hoxd9*, and *Hoxa11* were all expressed in the mesenchyme outside the elements, and each had at least some expression in the perichondrium (Figure 1D). *Shox2* and *Hoxa11* were additionally each expressed in the proliferating chondrocytes of the elements in their respective expression domains (Figure 1D).

Given that the mutations of *Shox2* or *Hox* genes similarly give rise to regional phenotypes and that they have closely associated expression dynamics, we sought to determine whether these genes might function together during limb development. Mice harboring *Shox2* mutations (Cobb *et al.* 2006), a complete deficiency of the *HoxD* cluster (Spitz *et al.* 2001), and a *Cre*-dependent deletion of the entire *HoxA* cluster (Kmita *et al.* 2005) were crossed, and the limbs of resultant progeny were analyzed for evidence of epistasis as newborns (Figure 2). Intriguingly, while the removal of an individual *Shox2* allele did not lead to limb shortening when the *Hox* genes were intact, this same mutation gave significantly truncated humeri when the *HoxD* cluster was disrupted (~30% shorter than expected if *HoxD* genes did not affect the outcome of *Shox2* mutation) [Figure 2, A and A′: nonparallel lines in interaction plots are characteristic of interaction (*e.g.*, Zhu *et al.* 2014)]. Additionally, *Prrx1-Cre*; $Shox2^{f/-}$ (or $Shox2^{c/-}$) animals, which have both copies of *Shox2* removed from the limb mesenchyme, have disproportionately shorter ulnae when the *HoxA* genes are concomitantly disrupted (~25% shorter than expected if

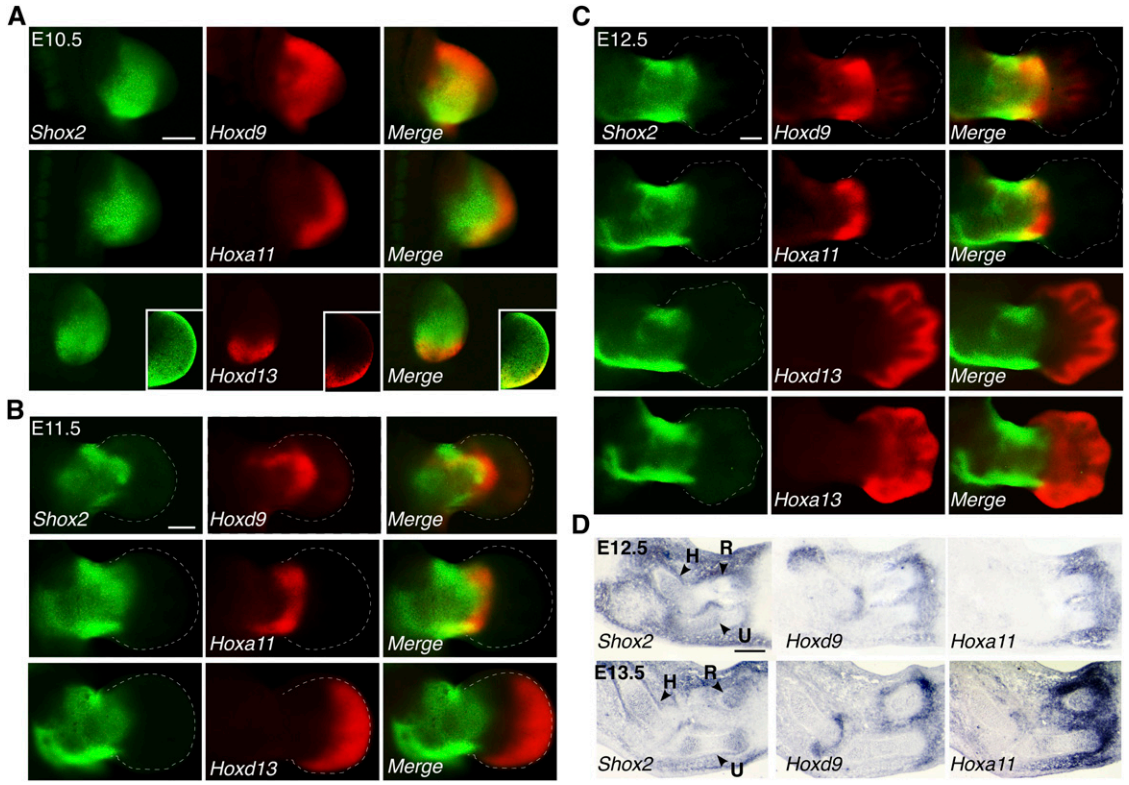

Figure 1 Characterization of *Shox2* and *Hox* gene expression. (A–C) Fluorescence *in situ* hybridization of *Shox2* and *Hox* genes in single embryos, with yellow signal in the merged image showing coexpression. (A) At E10.5, *Hox* genes are expressed in a nested pattern within the *Shox2* expression domain, which occupies the majority of the limb bud. The inset for the *Shox2*/*Hoxd13* series shows a single optical section through the limb bud, using confocal microscopy. (B) At E11.5, *Shox2* expression is confined to the proximal limb and shares a similar distal expression border to that of the *Hoxd9* and *Hoxa11* expression domains. *Hoxd13* is exclusively expressed in the distal limb bud. (C) At E12.5, *Shox2* expression is maintained in the proximal limb. *Hoxd9* and *Hoxa11* are expressed in the zeugopod, with *Hoxd9* additionally being expressed in the developing digits. *Hoxd13* is expressed in the developing digits, and *Hoxa13* is expressed in the developing digits and carpals. (D) Chromogenic *in situ* hybridization at E12.5 and E13.5 shows *Shox2* and the *Hox* genes are expressed both outside and within the elements of the stylopod and zeugopod. *Shox2* and *Hoxa11* are each expressed in the proliferating chondroctyes within their respective expression domains. Expression outlining each element corresponds to the perichondrium. H, humerus; R, radius; U, ulna. Bar, 0.25 mm.

HoxA genes did not affect the outcome of *Shox2* mutation) (Figure 2, B and B′). Thus, sensitized *Hox* backgrounds reveal dose-dependent roles for *Shox2* in the growth of limb elements, including a previously unrecognized yet sizable role in the growth of the newborn zeugopod. To verify these results in a statistical framework and also extend the analysis to each element of the stylopod and zeugopod of both the fore- and hindlimbs, we used a linear model (ANOVA) to identify significant interactions between *Shox2*, *HoxA*, and *HoxD* alleles (see *Materials and Methods*) (Supporting Information, Table S1). This analysis revealed epistasis between *Shox2* and *Hox* genes in all elements of the stylopod and zeugopod (with the exception of the developing fibula), where there were significant differences between the observed bone lengths and those expected if there were no interactions (shown as "estimates" in Table S2). These interactions were associated with entire series of genotypes and were often similar for corresponding skeletal elements of the fore- and hindlimbs (Figure 2, A′′ and B′′). Overall, there is pervasive epistasis between loss-of-function alleles of *Shox2* and *Hox* genes, where modulating their relative gene dose produces graded changes in the lengths of individual skeletal elements.

To further analyze the role of *Shox2* in limb growth, we also examined the effects of *Shox2* gain-of-function on the developing limb. Does *Shox2* overexpression lead to limb lengthening, as has been proposed for human *SHOX*? Or, as *Shox2* and *Hox* genes function together, can *Shox2* overexpression compensate for the loss of *Hox* gene function? To address these questions, a line of mice with a single copy of *Shox2* targeted to the *Rosa26* locus (Scott *et al.* 2011) was crossed to *Prrx1-Cre* mice, giving rise to embryos with ectopic *Shox2* expression throughout the mesenchyme of the developing limb (Figure 3A) (referred to as "*RosaShox2*" animals). To define the relative level of *Shox2* expression in these mice, entire E10.5 limb buds and proximal E12.5 limb buds were microdissected, and quantitative real-time polymerase chain reaction (RT-PCR) was performed. For each stage, there was an average increase of between 2 and 2.5 times compared to wild type (Figure 3B). Analysis of *RosaShox2* hemizygotes failed to show any limb lengthening and actually showed a slightly reduced humeral

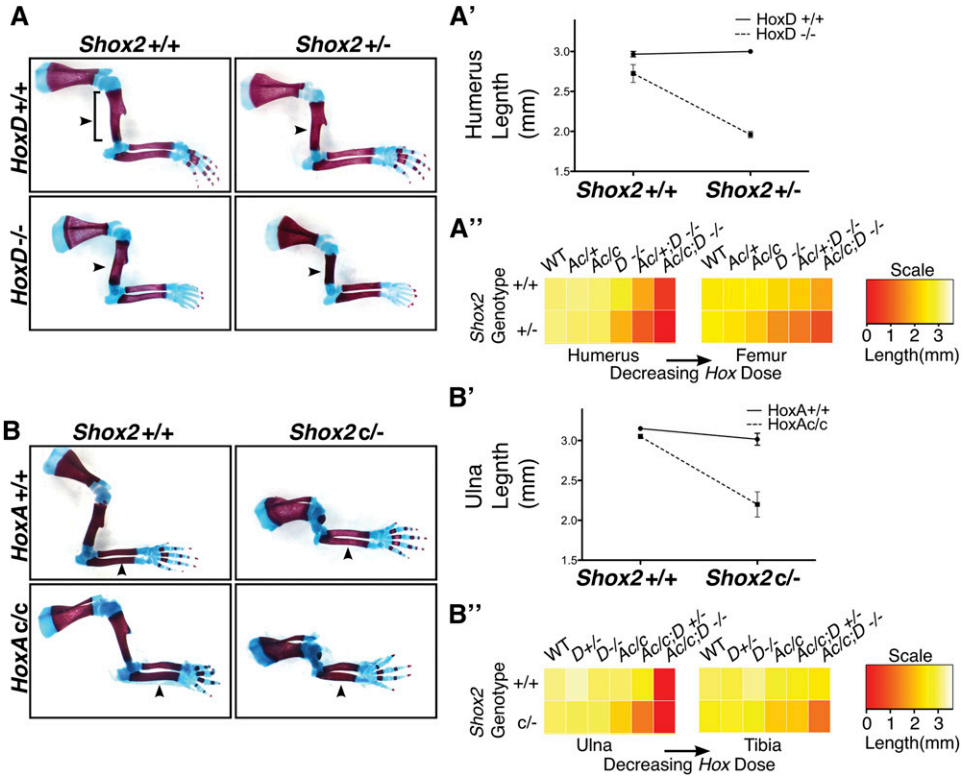

Figure 2 Epistasis between *Shox2* loss-of-function mutations and *Hox* genes during growth of the stylopodal and zeugopodal elements. (A) Deletion of a single copy of *Shox2* has no effect on the newborn limb in an otherwise wild-type background, but leads to a shortened humerus in a *HoxD*$^{-/-}$ background (n = 4–6 for each genotype). Arrowhead points to humerus. (B) Deletion of both copies of *Shox2* has a stronger effect on the growth of the ulna in a *HoxA*$^{c/c}$ background than in an otherwise wild-type background (n = 4–6 for each genotype). Arrowhead points to ulna. (A′ and B′) Interaction plots showing disproportionate effects, as shown by nonparallel lines, of the respective *Shox2* mutation in each *Hox* background. Data are plotted as means ± SEM. Effect of *Shox2* mutation is significantly different in each *Hox* background compared to an otherwise wild-type background. $P <$ 0.001. (A′′ and B′′) Genotype–phenotype maps showing the effects of *Shox2* mutations on skeletal length in multiple *Hox* backgrounds, arranged in decreasing dose, in both the fore- and hindlimbs.

length compared to wild type (Figure 3, C and C′). These animals furthermore lacked lateral ossifications in the autopod and were missing their scapular spine. The *RosaShox2* allele could, importantly, functionally replace the native *Shox2* gene, as *RosaShox2*; *Shox2*$^{c/-}$ animals had humeri of similar lengths to those of wild-type and *RosaShox2* animals (Figure 3, C and C′). We crossed the *RosaShox2* allele into the *Hox* cluster-deficient lines to generate animals that overexpress *Shox2* in each *Hox* mutant background, measured the lengths of the limb segments in the progeny, and incorporated the data into our limb models. This analysis revealed that the *RosaShox2* allele could extend the length of the humerus of the two most severe *Hox* mutants, although in a limited manner (Figure 3C′) (Table S2), indicating *Shox2* overexpression can partially compensate for *Hox* gene loss. We further found that, in a *HoxA/D* mutant background, graded modulation of effective *Shox2* levels through under- and overexpression gave stepwise changes in humerus length (Figure 3D). Thus, *Shox2* is sufficient to drive humerus growth in the absence of the *HoxA* and *HoxD* clusters, but only in a limited and inefficient manner. These data suggest that *Shox2* and *Hox* gene functions normally synergize to drive the robust growth of the humerus.

To investigate the cellular basis of the limb shortening in *Shox2* and *Hox* mutants, we went on to examine the expression of markers and regulators of chondrogenesis. Previous studies have demonstrated a delay in humeral chondrogenesis, associated with a loss or reduction in *Runx2* levels, when *Shox2* is deleted in the limb mesenchyme (Cobb *et al.* 2006; Yu *et al.* 2007). To ascertain whether *Shox2* and *Hox* mutants have a similar delay in chondrogenesis, we therefore examined *Col2a1*, *Runx2*, and *Col10a1* expression, in wild-type, *Shox2*$^{c/-}$, and *HoxA*$^{c/c}$; *HoxD*$^{-/-}$ animals (Figure 4A). Sections at E14.5, when chondrogenesis is well underway, showed that both wild-type and mutant humeri contained *Col2a1*-positive cells, with wild-type animals additionally showing down regulation in the middle of the element, corresponding to the zone of maturation. *Runx2* was strongly expressed in the chondrocytes and the perichondrium of wild-type animals, but this expression was depressed in the humeri of both *Shox2* and *Hox* mutants. Furthermore, wild-type animals showed strong *Col10a1* expression in the middle of the humerus, which was also absent in both *Shox2*$^{c/-}$ and *HoxA*$^{c/c}$; *HoxD*$^{-/-}$ animals. These data suggest that *Shox2* and *Hox* mutants fail to undergo timely chondrocyte maturation. In support of this view, hematoxylin and eosin staining showed that wild-type humeri contained hypertrophic chondrocytes in the middle of the element, while *Shox2* and *Hox* mutants displayed only immature chondrocytes (Figure 4B). Thus the coordinated action of *Shox2* and *Hox* genes likely drives cartilage maturation upstream of *Runx2* during development.

Given that *Shox2* and *Hox* genes functionally interact and have associated expression dynamics, we considered that they might influence one another's transcription. In agreement with earlier studies (Cobb *et al.* 2006; Yu *et al.* 2007), no changes in *Hox* gene expression were found in *Shox2*$^{c/-}$ animals (data not shown), suggesting *Shox2* does not function upstream of *Hox* transcription. Considering the converse relationship, we examined whether *Hox* genes may activate

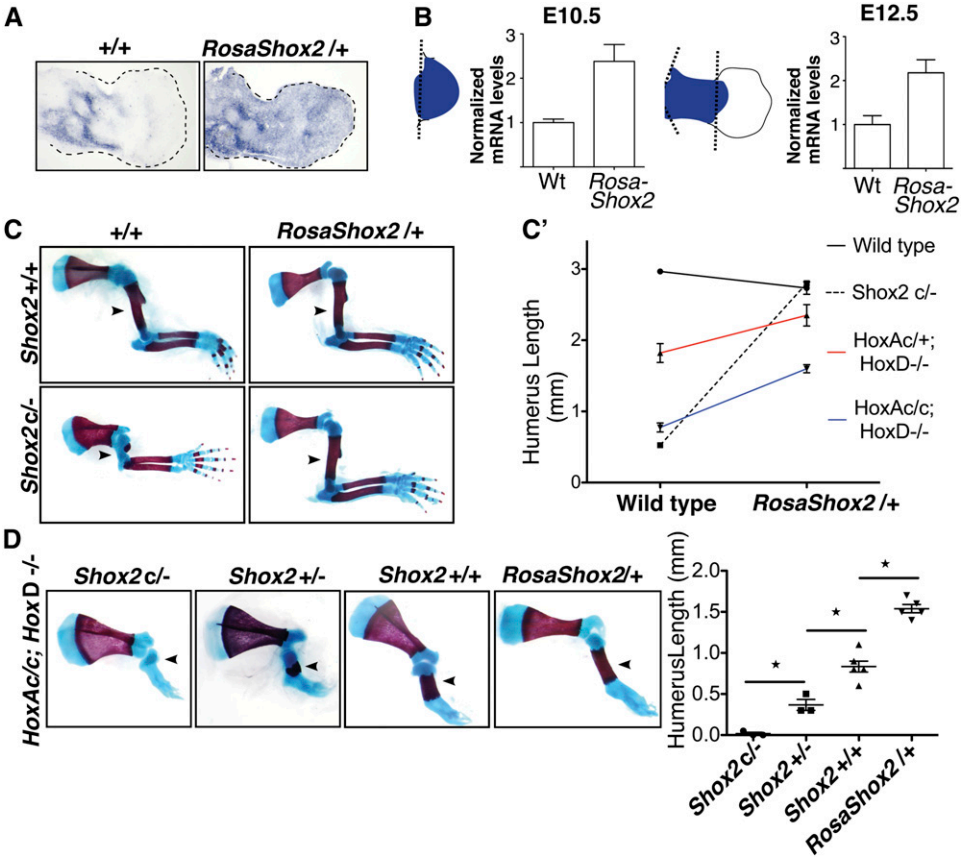

Figure 3 Epistasis between *Shox2* gain-of-function and *Hox* genes during growth of the stylopod. (A) *Shox2* ISH on wild-type and *RosaShox2* limbs at E12.5. (B) Real-time PCR determining the relative *Shox2* levels in wild-type and *RosaShox2* animals in whole E10.5 forelimb buds and proximal E12.5 forelimb buds. (C) Newborn forelimb skeletons showing the effects of *Shox2* overexpression in wild-type and $Shox2^{c/-}$ backgrounds. Arrowheads point to humerus. (C′) Interaction plot displaying the effects of overexpressing *Shox2* in wild-type, $Shox2^{c/-}$, $HoxA^{c/+}$; $HoxD^{-/-}$, and $HoxA^{c/c}$; $HoxD^{-/-}$ backgrounds (n = 3–6 for each genotype). Data are plotted as means ± SEM. Effect of *RosaShox2* is significantly different in each mutant background compared to an otherwise wild-type background. $P < 0.001$. (D) Newborn forelimb skeletons and quantification of the effect of modulating *Shox2* dose in a $HoxA^{c/c}$; $HoxD^{-/-}$ background (n = 3–6 for each genotype). Arrowheads point to humerus. $P < 0.05$.

Shox2 expression. *In situ* hybridization at E10.5, when there is maximal overlap between *Shox2* and *Hox* expression (Figure 1), revealed a reproducible dampening of *Shox2* transcript levels in $HoxA^{c/c}$; $HoxD^{-/-}$ animals, compared to wild type, throughout the limb bud (n = 3/3) (Figure 5). This regulation could reflect direct *trans*-activation by HOX proteins and/or indirect regulation through intermediary molecules. Supporting a contribution from the latter scenario, it was reported that *Shh*, which is activated by *Hox* function (Kmita *et al.* 2005), is required for normal *Shox2* transcript levels at E10.5 (Probst *et al.* 2011). This downregulation of *Shox2* expression is transient, however, as $HoxA^{c/c}$; $HoxD^{-/-}$ animals show similar *Shox2* expression at E11 to wild type, despite having a drastic difference in limb bud morphology (Figure 5). A recent report demonstrated that *Hox11* genes are required for *Shox2* expression in the proliferating chondrocytes of the zeugopodal elements (Gross *et al.* 2012), suggesting *Hox* genes could generally function upstream of *Shox2* expression in these cells. To assess whether *Hox* genes are necessary for *Shox2* activation in the developing humerus, ISH was performed on sections of wild-type and *HoxA/D* mutant limbs at E14.5. However, both wild-type and $HoxA^{c/c}$; $HoxD^{-/-}$ animals had similar *Shox2* expression in the humeral chondrocytes (n = 3/3) (Figure 5), suggesting any *Hox*-mediated transcriptional control of *Shox2* in this cell population is limited. There was, however, a lack of *Shox2* expression in the perichondrium of the humerus in *Hox* mutants (n = 3/3) (Figure 5). As the strongest perichondrial expression of *Shox2* in wild-type animals is adjacent to the zone of maturation (Figure 5), which $HoxA^{c/c}$; $HoxD^{-/-}$ animals lack, this absence of *Shox2* expression is likely, at least in part, a secondary consequence of a failure to undergo chondrocyte maturation. However, we also recently showed that chondrocyte-specific deletion of *Shox2* leads to a considerably less severe phenotype than its removal from the entire limb (Bobick and Cobb 2012), implicating the presence of additional *Shox2*-expressing cells during growth. Given the intimate association of the perichondrium with the underlying chondrocytes and their critical functional interactions (Kronenberg 2007), perichondrial *Shox2* expression represents a potential candidate for making contributions to limb growth. Thus, the absence of *Shox2* expression in the perichondrium of *Hox* mutants may indeed contribute to a lack of growth in these animals.

Discussion

This work investigates the genetic relationship between *Shox2* and *Hox* genes during the growth and development of the limb segments. We analyzed their relative expression dynamics during normal development, using representative *Hox* genes, showing they have associated expression changes that occur in broad domains along the proximodistal axis. We then manipulated the relative expression levels of Shox2 and Hox genes, demonstrating these adjustments tune the length of individual skeletal elements through

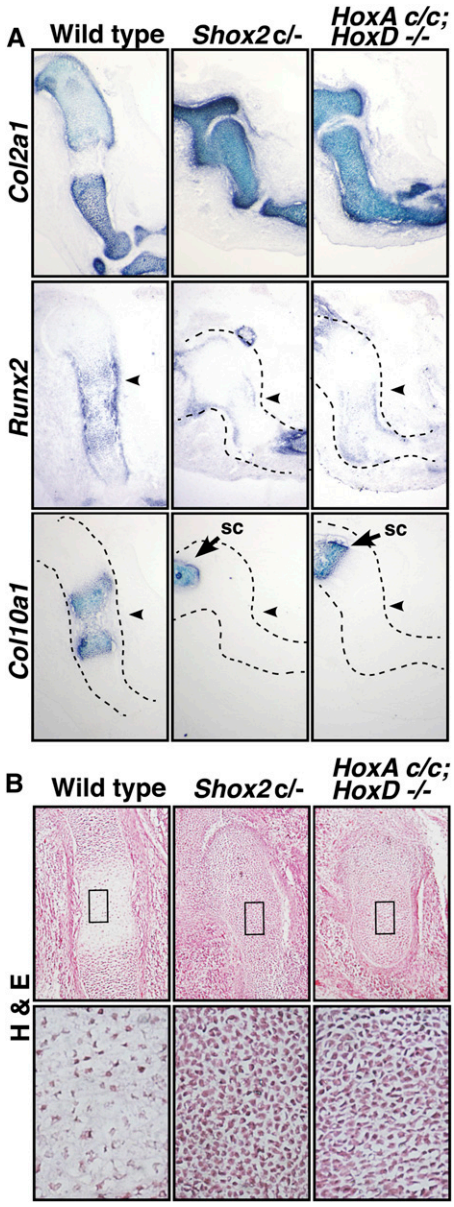

Figure 4 Independent mutation of *Shox2* and *Hox* genes results in a similar delay in humeral chondrocyte maturation. Shown are *in situ* hybridization and histological staining of E14.5 forelimb sections. (A) Wild-type, $Shox2^{c/-}$, and $HoxA^{c/c}$; $HoxD^{-/-}$ humeri all show *Col2a1* expression, with wild-type limbs additionally showing downregulation of expression in the middle of the element. Wild-type humeri have prominent *Runx2* and *Col10a1* expression, while $Shox2^{c/-}$ and $HoxA^{c/c}$; $HoxD^{-/-}$ animals show reduced *Runx2* levels and a lack of *Col10a1* expression. Arrowheads point to the middle of the humerus. sc, scapula. (B) Hematoxylin and eosin staining showing that wild-type limbs contain hypertrophic chondrocytes, while $Shox2^{c/-}$ and $HoxA^{c/c}$; $HoxD^{-/-}$ animals lack these cells.

epistatic interactions. Finally, we show that both *Shox2* and *Hox* genes function upstream of *Runx2* expression and are required for normal chondrocyte maturation.

Characterization of the relative gene expression patterns of *Shox2* and *Hox* genes showed that they are dynamic and occupy broad regions along the proximodistal axis. While *Shox2* is initially coexpressed with multiple *Hox* genes, it is restricted to the stylopodal and zeugopodal domains at later stages, along with *Hoxd9* and *Hoxa11*, and juxtaposes the distal expression of *Hoxd13* and *Hoxa13*. Thus, *Shox2* expression closely resembles the "early" phase of *Hox* expression (Tarchini and Duboule 2006; Andrey *et al.* 2013). Interestingly, it was recently demonstrated that a *Shox2-Cre* knock-in allele also drives reporter activity in a proximally restricted manner (Sun *et al.* 2013), suggesting the lineage of early *Shox2*-expressing cells does not contribute to digit development. Thus, while *Shox2* expression appears throughout the early limb bud (Figure 1A), it is likely not expressed in the presumptive digit cells. This view is consistent with a recent study showing fish *Hox* enhancers have activity in the "distal" part of the early mouse limb bud and that this domain later corresponds to the proximal limb (Woltering *et al.* 2014). The coincidence of *Shox2* and the early phase of *Hox* expression may reflect an ancestral functional relationship in the fins of fish, as both *Hox* genes and *Shox2* are expressed in the developing zebrafish fins (Sordino *et al.* 1995; Thisse and Thisse 2004).

It is currently unknown how the rodent evolutionary lineage tolerated the loss of *Shox*. However, given that *Shox* is otherwise conserved in vertebrates, expressed in the developing zeugopods of chicks and humans, and required for normal human limb development (Rao *et al.* 1997; Belin *et al.* 1998; Shears *et al.* 1998), its loss likely required compensatory changes. We suggest that multiple factors were involved, and one of them could have been expression of *Shox2* in the developing zeugopod. This proposal is based on multiple lines of evidence. First, mouse *Shox2* is expressed in both the developing stylopodal and zeugopodal regions (Cobb *et al.* 2006; Bobick and Cobb 2012), and since *Shox2* and *Shox* are homologous genes, this could have helped buffer the effects of *SHOX* loss. It has indeed been previously shown that a human *SHOX* knock-in allele is able to compensate for the loss of mouse *Shox2* in the developing forelimb (Liu *et al.* 2011). Interestingly, the reported *SHOX2* expression domains in chicks and humans are restricted to the stylopod (Clement-Jones *et al.* 2000; Tiecke *et al.* 2006), suggesting that the distally extended expression of mouse *Shox2* may be a derived feature in rodents. And second, although the individual mutation of mouse *Shox2* has little effect on the newborn zeugopod, its mutation in sensitized *Hox* backgrounds demonstrates its prominent function in this region. Based on this model, identifying additional factors that helped compensate for *Shox* loss could be an active area for future research.

Shox2 and *Hox* genes play critical roles in the formation of the limb skeleton, as evidenced by the strong phenotypes observed following their genetic ablation. Emerging evidence indicates that the activation of *Runx2* is a critical point of regulation by these genes (Cobb *et al.* 2006; Villavicencio-Lorini *et al.* 2010; Gross *et al.* 2012). The present work supports this general model and extends it by revealing that *Shox2* and *Hox* genes genetically interact during the development of both the stylopod and zeugopod elements. Given

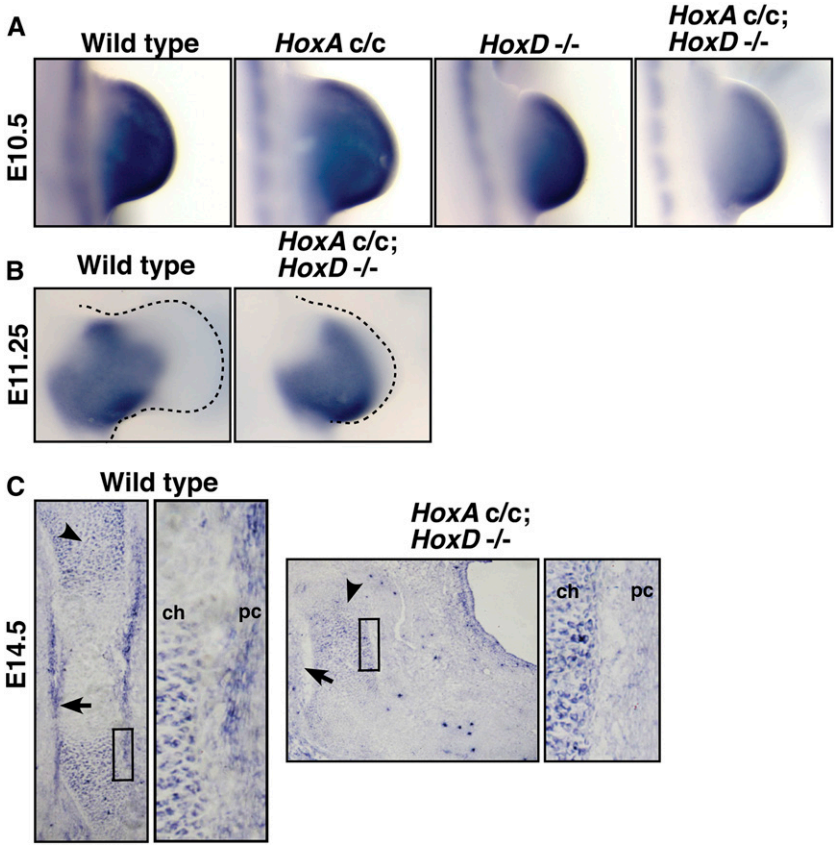

Figure 5 Disruption of *Hox* genes gives a transient decrease in *Shox2* expression in the early limb bud and a lack of *Shox2* expression in the perichondrium, as seen through *in situ* hybridization. (A) At E10.5, $HoxA^{c/c}$; $HoxD^{-/-}$ animals have a decrease in *Shox2* expression, compared to wild-type, $HoxA^{c/c}$, and $HoxD^{-/-}$ animals (n = 3/3). (B) At E11, $HoxA^{c/c}$; $HoxD^{-/-}$ animals have similar *Shox2* levels to those of wild-type controls (n = 2/2). (C) At E14.5, *Shox2* is expressed in the proliferating chondrocytes (arrowhead) and the perichondrium (arrow) of wild-type animals, but expression is selectively absent in the perichondrium of $HoxA^{c/c}$; $HoxD^{-/-}$ animals (n = 3/3). ch, chondrocytes; pc, perichondrium.

the similar regional shortening in the limbs of humans with *SHOX* mutations, we furthermore propose that *Shox* and *Hox* genes may generally coordinate *Runx2* expression in the stylopodal and zeugopodal elements of vertebrate limbs.

Our analysis extensively investigated the genetic relationship of *Shox* and *Hox* genes, revealing multiple interesting features. When these genes are independently and fully deleted, they give prominent phenotypes, although they have some ability to tolerate intermediate changes in dose. These relatively "steep" phenotypic responses are in contrast to the changes that occur from the concomitant disruption of *Shox2* and *Hox* genes. In these cases, the limb is much more sensitive to *Shox2* and *Hox* disruption, giving less drastic, yet more widespread, changes in phenotype. These genetic interactions thus normally confer the ability to buffer the effects of some variation in gene product levels. Such perspectives provide insight into how the level of robustness of a system influences its response to mutation and is relevant to the establishment of genetic disease. In this regard, it is interesting to note that both human *SHOX* and human *HOXA11* are considered haplo-insufficient loci (Rao *et al.* 1997; Thompson and Nguyen 2000), suggesting the zeugopod genetic network they function in may be somewhat sensitive to mutation in general. While the sources of the dosage sensitivity in *Hox–Shox* networks are unknown, it may furthermore extend to *Runx2* regulation, as disruption of a single *Runx2* allele causes limb defects (Otto *et al.* 1997). We also extensively explored the relationship between under- and overexpression of *Shox2*. In both wild-type and many *Hox* mutant backgrounds, *Shox2* overexpression did not extend limb length, suggesting *Shox2* levels were already present in excess or "saturating". This was in contrast to the most severe *Hox* backgrounds, where the humerus responded nearly linearly to deletions of *Shox2* and, conversely, *Shox2* overexpression gave significant gains in length. Thus, only in those backgrounds where the limb was most sensitive to *Shox2* reductions was the humerus also responsive to elevated *Shox2* levels. In addition to providing insight into the constancy of developmental phenotypes, these data may be furthermore relevant to the effects of human *SHOX* dosage. It is hypothesized that *SHOX* overexpression leads to tall stature in humans, which is based on the observation that *SHOX* deletions are dosage sensitive and that people with three or more sex chromosomes (and therefore supernumerary copies of *SHOX*, as *SHOX* resides on the pseudoautosomal region of the sex chromosomes) are significantly taller than expected (Durand and Rappold 2013). However, associated with chromosome aneuploidies are changes in hormone levels, which influence the maturation of the growth plate during longitudinal bone growth (Smith *et al.* 1994; Morishima *et al.* 1995), thus confounding interpretations of the cause of tall stature (Ottesen *et al.* 2010). As *SHOX* deletions are dosage sensitive, *SHOX* overexpression alone may very well lead to longer limbs, but we also suggest there are likely limitations to its effects. Available data suggest that the response to *SHOX* deletions is

quite nonlinear, where individuals with two deleted copies have a much more severe phenotype than those with a deletion in a single copy (Belin *et al.* 1998; Shears *et al.* 1998; Schiller *et al.* 2000; Zinn *et al.* 2002; Albuisson *et al.* 2012), suggesting the amount of functional *SHOX* levels may be approaching saturation in people with two intact copies. Thus, any substantial role *SHOX* overexpression has in overgrowth may be in conjunction with associated changes of having additional sex chromosomes, in line with the hypothesis that *SHOX* overexpression interacts with hormonal changes (Ogata *et al.* 2000).

The possibility that *Hox* genes regulate *Shox2* expression was examined in considerable detail. We found that the *HoxA/D* clusters are required for full activation of *Shox2* early on, but this regulation on its own may be of limited consequence as it is transient. However, given that *Hox* genes likely have multiple roles during limb development, even a transient decrease in *Shox2* expression could make contributions to quantitative *Hox* phenotypes. Mutation of *Hox* genes additionally resulted in the loss of *Shox2* expression in the perichondrium of the developing humerus, which was associated with a failure to undergo chondrocyte maturation. Future experiments are required to address any causal role of perichondrial *Shox2* expression in driving the growth of the humerus or whether this expression is involved in later steps of skeletogenesis, such as the generation of bone. As *Hox* genes are also hypothesized to exert a large part of their effect on the developing skeleton from their expression in the perichondrium (Villavicencio-Lorini *et al.* 2010; Swinehart *et al.* 2013), their cell autonomous role in this structure should also be investigated. Of note, although *Hox* genes are required for normal *Shox2* expression, such regulation cannot completely account for the role of *Hox* genes in growth, as *Shox2* overexpression failed to fully rescue the *Hox* mutant phenotype. Thus, interactions between *Shox2* and *Hox* genes during mouse limb development likely involve diverse mechanisms, which could also be the case for any interactions between human *SHOX* and *HOX* genes. Elucidation of the biochemical mechanism underlying SHOX and HOX cooperativity will be of considerable interest. SHOX and HOX proteins could, for example, coordinately bind common enhancers, bind distinct enhancers that synergistically activate transcription, or regulate different target genes that themselves act in a cooperative manner. Thus, identifying genomic regions that are bound by SHOX2 and HOX proteins during limb development could help illuminate the basis of their genetic interaction.

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GENETICS

Supporting Information
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Genetic Interactions Between *Shox2* and *Hox* Genes During the Regional Growth and Development of the Mouse Limb

Stanley J. Neufeld, Fan Wang, and John Cobb

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Table S1 ANOVA results modeling the effect of *Shox2*, *HoxA,* and *HoxD* function on the growth of each skeletal element of the zeugopod and stylopod. Df = degrees of freedom; SS = sum of squares (Type III); F = Ratio of the between group and within group variability. Pr(>F) = Probability of obtaining the respective F statistic under the null hypothesis (*p*-value).

Model:
Humerus Length ~ Shox2 * HoxA * HoxD
(read as humerus length modeled as a function of *Shox2* interacting with *HoxA* interacting with *HoxD*)

	Df	SS	F	Pr(>F)
Shox2	3	126.833	2894.688	< 2.2e-16
HoxA	2	5.257	179.960	< 2.2e-16
HoxD	2	30.027	1027.947	< 2.2e-16
Shox2: HoxA	6	1.550	17.684	2.133e-14
Shox2: HoxD	6	6.950	79.315	< 2.2e-16
HoxA: HoxD	4	5.828	99.750	< 2.2e-16
Shox2: HoxA: HoxD	12	2.782	15.873	< 2.2e-16

Model:
Ulna Length ~ Shox2 * HoxA * HoxD

	Df	SS	F	Pr(>F)
Shox2	3	5.5549	60.0193	< 2.2e-16
HoxA	2	7.2493	117.4913	< 2.2e-16
HoxD	1	0.9520	30.8586	3.711e-07
Shox2: HoxA	6	3.4583	18.6834	2.407e-13
Shox2: HoxD	3	0.2692	2.9086	0.03978
HoxA: HoxD	2	1.0646	17.2547	6.242e-07
Shox2: HoxA: HoxD	6	0.3595	1.9421	0.08435

Model:
Radius Length ~ Shox2 * HoxA * HoxD

	Df	SS	F	Pr(>F)
Shox2	3	0.6928	7.9367	0.00011
HoxA	2	4.9228	84.5900	< 2.2e-16
HoxD	1	1.2071	41.4844	8.935e-09
Shox2: HoxA	6	0.9697	5.5541	7.788e-05
Shox2: HoxD	3	0.0700	0.8021	0.49645
HoxA: HoxD	2	1.1679	20.0685	9.303e-08
Shox2: HoxA: HoxD	6	0.3828	2.1925	0.05243

Model:

Femur Length ~ Shox2 * HoxA * HoxD

	Df	SS	F	Pr(>F)
Shox	2	3.0718	71.5967	< 2.2e-16
HoxA	2	0.5685	13.2495	9.969e-06
HoxD	2	4.3596	101.6109	< 2.2e-16
Shox: HoxA	4	0.6202	7.2279	4.731e-05
Shox: HoxD	4	2.3454	27.3326	1.544e-14
HoxA: HoxD	4	0.2961	3.4506	0.01163
Shox: HoxA: HoxD	8	0.1533	0.8934	0.52585

Model:

Tibia Length ~ Shox2 * HoxA * HoxD

	Df	SS	F	Pr(>F)
Shox2	3	5.1478	53.3577	< 2.2e-16
HoxA	2	5.5804	86.7621	< 2.2e-16
HoxD	2	1.4070	21.8748	9.328e-09
Shox2: HoxA	6	2.5650	13.2932	2.378e-11
Shox2: HoxD	6	0.1280	0.6634	0.67932

HoxA: HoxD	4	1.0833	8.4214	5.538e-06
Shox2: HoxA: HoxD	12	0.8549	2.2154	0.01529

Model:

Fibula Length ~ Shox2 * HoxA * HoxD

	Df	SS	F	Pr(>F)
Shox2	3	1.52652	18.2074	1.046e-09
HoxA	2	1.07932	19.3101	6.112e-08
HoxD	2	0.21036	3.7636	0.0261584
Shox2: HoxA	6	0.79354	4.7325	0.0002459
Shox2: HoxD	6	0.12233	0.7295	0.6267508
HoxA: HoxD	4	0.15297	1.3684	0.2493868
Shox2: HoxA: HoxD	12	0.34533	1.0297	0.4269231

Table S2 Results of contrasts showing differences between expected and observed skeletal lengths for each *Shox2* mutation in wild-type vs. *Hox*-mutant backgrounds. To control for Type I error, a Bonferroni correction was applied to the data sets for each skeletal element. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Genotype	Estimate (mm)	Std. Error	*p* (unadjusted)	*p* (Bonferroni adjusted)
Humerus				
Shox2+/- by HoxA+/c	0.125	0.116	0.282	1.000
Shox2+/- by HoxD+/-	0.233	0.110	0.037*	0.879
Shox2+/- by HoxA+/c;HoxD+/-	0.292	0.116	0.013*	0.314
Shox2+/- by HoxAc/c	0.083	0.110	0.452	1.000
Shox2+/- by HoxD-/-	0.798	0.107	1.81E-11***	4.34E-10***
Shox2+/- by HoxAc/c;HoxD+/-	0.467	0.110	4.74E-05***	1.14E-03**
Shox2+/- by HoxA+/c;HoxD-/-	0.747	0.113	1.13E-09***	2.72E-08***
Shox2+/- by HoxAc/c;HoxD-/-	0.500	0.110	1.45E-05***	3.48E-04***
Shox2c/- by HoxA+/c	0.050	0.110	0.651	1.000
Shoxc/- by HoxD+/-	0.050	0.110	0.651	1.000
Shox2c/- by HoxA+/c;HoxD+/-	0.208	0.116	0.074	1.000
Shox2c/- by HoxAc/c	0.100	0.110	0.367	1.000
Shox2c/- by HoxD-/-	0.150	0.110	0.177	1.000
Shox2c/- by HoxAc/c;HoxD+/-	0.220	0.101	0.032*	0.759
Shox2c/- by HoxA+/c;HoxD-/-	-0.520	0.107	3.76E-06***	9.01E-05***
Shox2c/- by HoxAc/c;HoxD-/-	-1.583	0.110	< 2e-16 ***	< 2e-16***
RosaShox2 by HoxA+/c	-0.025	0.116	0.829	1.000
RosaShox2 by HoxD+/-	0.183	0.110	0.099	1.000
RosaShox2 by HoxA+/c;HoxD+/-	0.017	0.121	0.891	1.000
RosaShox2 by HoxAc/c	-0.117	0.110	0.293	1.000
RosaShox2 by HoxD-/-	0.075	0.116	0.518	1.000
RosaShox2 by HoxAc/c;HoxD+/-	-0.067	0.105	0.525	1.000
RosaShox2 by HoxA+/c;HoxD-/-	-0.670	0.113	2.94E-08***	7.06E-07***
RosaShox2 by HoxAc/c;HoxD-/-	-0.923	0.101	2.89E-15***	6.93E-14***

Ulna

Shox2+/- by HoxA+/c	0.058	0.168	0.730	1.000
Shox2+/- by HoxD+/-	0.267	0.160	0.100	1.000
Shox2+/- by HoxA+/c;HoxD+/-	0.083	0.168	0.622	1.000
Shox2+/- by HoxAc/c	0.267	0.160	0.100	1.000
Shox2+/- by HoxAc/c;HoxD+/-	0.383	0.160	0.019*	0.288
Shox2c/- by HoxA+/c	0.267	0.160	0.100	1.000
Shoxc/- by HoxD+/-	0.242	0.160	0.136	1.000
Shox2c/- by HoxA+/c;HoxD+/-	0.308	0.168	0.071	1.000
Shox2c/- by HoxAc/c	0.717	0.160	2.62E-05***	3.93E-04***
Shox2c/- by HoxAc/c;HoxD+/-	1.297	0.147	2.37E-13***	3.56E-12***
RosaShox2 by HoxA+/c	0.175	0.168	0.301	1.000
RosaShox2 by HoxD+/-	0.383	0.160	0.019*	0.288
RosaShox2 by HoxA+/c;HoxD+/-	0.217	0.176	0.221	1.000
RosaShox2 by HoxAc/c	-0.042	0.160	0.796	1.000
RosaShox2 by HoxAc/c;HoxD+/-	0.008	0.152	0.957	1.000

Radius

Shox2+/- by HoxA+/c	0.258	0.163	0.118	1.000
Shox2+/- by HoxD+/-	0.392	0.156	0.014*	0.209
Shox2+/- by HoxA+/c;HoxD+/-	0.192	0.163	0.244	1.000
Shox2+/- by HoxAc/c	0.242	0.156	0.125	1.000
Shox2+/- by HoxAc/c;HoxD+/-	0.333	0.156	0.035*	0.531
Shox2c/- by HoxA+/c	0.208	0.156	0.185	1.000
Shoxc/- by HoxD+/-	0.083	0.156	0.594	1.000
Shox2c/- by HoxA+/c;HoxD+/-	0.108	0.163	0.509	1.000
Shox2c/- by HoxAc/c	0.258	0.156	0.101	1.000
Shox2c/- by HoxAc/c;HoxD+/-	0.597	0.143	7.53E-05***	1.13E-03**
RosaShox2 by HoxA+/c	0.208	0.163	0.206	1.000
RosaShox2 by HoxD+/-	0.333	0.156	0.035	0.531
RosaShox2 by HoxA+/c;HoxD+/-	0.083	0.171	0.627	1.000
RosaShox2 by HoxAc/c	-0.042	0.156	0.790	1.000
RosaShox2 by HoxAc/c;HoxD+/-	-0.108	0.148	0.466	1.000

Femur

Shox2+/- by HoxA+/c	0.125	0.140	0.375	1.000
Shox2+/- by HoxD+/-	0.225	0.134	0.096	1.000
Shox2+/- by HoxA+/c;HoxD+/-	0.317	0.140	0.027*	0.424
Shox2+/- by HoxAc/c	0.350	0.134	0.010*	0.168
Shox2+/- by HoxD-/-	0.690	0.130	8.36E-07***	1.34E-05***
Shox2+/- by HoxAc/c;HoxD+/-	0.483	0.134	5.11E-04***	8.18E-03**
Shox2+/- by HoxA+/c;HoxD-/-	0.783	0.136	1.43E-07***	2.29E-06***
Shox2+/- by HoxAc/c;HoxD-/-	0.850	0.134	1.02E-08***	1.63E-07***
RosaShox2 by HoxA+/c	-0.058	0.140	0.678	1.000
RosaShox2 by HoxD+/-	0.050	0.134	0.709	1.000
RosaShox2 by HoxA+/c;HoxD+/-	0.067	0.147	0.650	1.000
RosaShox2 by HoxAc/c	0.000	0.134	1.000	1.000
RosaShox2 by HoxD-/-	0.000	0.140	1.000	1.000
RosaShox2 by HoxAc/c;HoxD+/-	-0.067	0.127	0.601	1.000
RosaShox2 by HoxA+/c;HoxD-/-	-0.267	0.136	0.054	0.861
RosaShox2 by HoxAc/c;HoxD-/-	-0.483	0.127	2.63E-04***	4.21E-03**

Tibia

Shox2+/- by HoxA+/c	0.258	0.172	0.135	1.000
Shox2+/- by HoxD+/-	0.342	0.164	0.039*	0.939
Shox2+/- by HoxA+/c;HoxD+/-	0.167	0.172	0.334	1.000
Shox2+/- by HoxAc/c	0.242	0.164	0.143	1.000
Shox2+/- by HoxD-/-	0.162	0.159	0.311	1.000
Shox2+/- by HoxAc/c;HoxD+/-	0.267	0.164	0.106	1.000
Shox2+/- by HoxA+/c;HoxD-/-	0.187	0.167	0.266	1.000
Shox2+/- by HoxAc/c;HoxD-/-	0.317	0.164	0.056	1.000
Shox2c/- by HoxA+/c	0.125	0.164	0.447	1.000
Shoxc/- by HoxD+/-	0.175	0.164	0.287	1.000
Shox2c/- by HoxA+/c;HoxD+/-	0.075	0.172	0.663	1.000
Shox2c/- by HoxAc/c	0.450	0.164	0.007**	0.167
Shox2c/- by HoxD-/-	-0.175	0.164	0.287	1.000
Shox2c/- by HoxAc/c;HoxD+/-	0.463	0.150	2.54E-03**	0.06

Shox2c/- by HoxA+/c;HoxD-/-	-0.030	0.159	0.850	1.000
Shox2c/- by HoxAc/c;HoxD-/-	0.967	0.164	3.78E-08***	9.08E-07***
RosaShox2 by HoxA+/c	-0.125	0.172	0.468	1.000
RosaShox2 by HoxD+/-	0.225	0.164	0.172	1.000
RosaShox2 by HoxA+/c;HoxD+/-	-0.050	0.179	0.781	1.000
RosaShox2 by HoxAc/c	0.025	0.164	0.879	1.000
RosaShox2 by HoxD-/-	0.058	0.172	0.735	1.000
RosaShox2 by HoxAc/c;HoxD+/-	-0.092	0.155	0.556	1.000
RosaShox2 by HoxA+/c;HoxD-/-	-0.197	0.167	0.241	1.000
RosaShox2 by HoxAc/c;HoxD-/-	-0.225	0.155	0.150	1.000

Fibula

Shox2+/- by HoxA+/c	0.100	0.160	0.533	1.000
Shox2+/- by HoxD+/-	0.192	0.153	0.212	1.000
Shox2+/- by HoxA+/c;HoxD+/-	0.150	0.160	0.351	1.000
Shox2+/- by HoxAc/c	0.167	0.153	0.277	1.000
Shox2+/- by HoxD-/-	0.197	0.148	0.187	1.000
Shox2+/- by HoxAc/c;HoxD+/-	0.250	0.153	0.104	1.000
Shox2+/- by HoxA+/c;HoxD-/-	0.353	0.156	0.025*	0.602
Shox2+/- by HoxAc/c;HoxD-/-	0.017	0.153	0.913	1.000
Shox2c/- by HoxA+/c	0.058	0.153	0.703	1.000
Shoxc/- by HoxD+/-	-0.058	0.153	0.703	1.000
Shox2c/- by HoxA+/c;HoxD+/-	-0.050	0.160	0.755	1.000
Shox2c/- by HoxAc/c	0.242	0.153	0.116	1.000
Shox2c/- by HoxD-/-	-0.108	0.153	0.479	1.000
Shox2c/- by HoxAc/c;HoxD+/-	0.257	0.140	0.069	1.000
Shox2c/- by HoxA+/c;HoxD-/-	-0.163	0.148	0.272	1.000
Shox2c/- by HoxAc/c;HoxD-/-	0.283	0.153	0.066	1.000
RosaShox2 by HoxA+/c	0.083	0.160	0.604	1.000
RosaShox2 by HoxD+/-	0.225	0.153	0.143	1.000
RosaShox2 by HoxA+/c;HoxD+/-	0.017	0.167	0.921	1.000
RosaShox2 by HoxAc/c	0.075	0.153	0.624	1.000
RosaShox2 by HoxD-/-	0.200	0.160	0.214	1.000
RosaShox2 by HoxAc/c;HoxD+/-	0.200	0.145	0.170	1.000

RosaShox2 by HoxA+/c;HoxD-/-	-0.030	0.156	0.848	1.000
RosaShox2 by HoxAc/c;HoxD-/-	-0.042	0.145	0.774	1.000