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Identification of spontaneous mutations within the long-range limb-specific *Sonic Hedgehog* enhancer (*ZRS*) that alter *Sonic Hedgehog* expression in the chicken limb mutants *oligozeugodactly* and Silkie Breed

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Abstract

The evolutionarily conserved, non-coding ~800 base-pair zone of polarizing activity (ZPA) regulatory sequence (*ZRS*) controls *Shh* expression in the posterior limb. We report that the chicken mutant *oligozeugodactly (ozd*), which lacks limb *Shh* expression, has a large deletion within the *ZRS*. Furthermore, the preaxial polydactylous, Silkie Breed chicken, which develops ectopic anterior limb *Shh* expression, has a single base-pair change within the *ZRS*. Using an *in vivo* reporter assay to examine enhancer function in the chick limb, we demonstrate that the wild-type *ZRS* drives β-galactosidase reporter expression in the ZPA of both wild-type and *ozd* limbs. The Silkie ZRS drives β-galactosidase in both posterior and anterior *Shh* domains in wild-type limb buds. These results support the hypothesis that the *ZRS* integrates positive and negative prepatterned regulatory inputs in the chicken model system and demonstrate the utility of the chicken limb as an efficient genetic system for gene regulatory studies.

Keywords

limb development; limb bud prepattern; Sonic hedgehog (*SHH*); *LMBR1*; *oligozeugodactyly (ozd)*; zone of polarizing activity (ZPA); cis-enhancer; reporter analysis; ZPA regulatory sequence (*ZRS*); mammals-fishes-conserved-sequence-1 (*MFCS-1*); Acheiropodia (ACHP); preaxial polydactyly (PPD); Dorking Breed chicken; Silkie Breed chicken

INTRODUCTION

Patterning along the anteroposterior (A/P) axis of the developing vertebrate limb is controlled by asymmetric expression of the *Sonic hedgehog* (*Shh*) gene (reviewed in Towers and Tickle, 2009; Zeller et al., 2009). *Shh* expression is restricted to a specific region of mesenchyme along the border of the posterior limb bud, corresponding to the zone of polarizing activity (ZPA). Misregulation of *Shh* expression in the limb bud occurs and

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results in preaxial polydactyly (PPD) (Chan et al., 1995; Masuya et al., 1995; Masuya et al., 1997; Sharpe et al., 1999), or other abnormalities (Krebs et al., 2003). The genetic characterization of animal models with limb defects has permitted an understanding of the underlying mechanisms surrounding some of these disorders, as well as insights into the molecular mechanisms of normal limb development.

A highly conserved long-range limb-specific *Shh* enhancer is present within intron 5 of the *LMBR1* gene, located approximately 1 megabase upstream of the *Shh* gene (Lettice et al., 2002; Lettice et al., 2003; Sagai et al., 2004). This enhancer is called the ZPA regulatory sequence (*ZRS*) (Lettice et al., 2003) and is also known as mammals-fishes-conserved-sequence-1 (MFCS-1) (Sagai et al., 2005). The *ZRS* is syntenic with the *Shh* locus in all tetrapod species, teleost fish (Sagai et al., 2004), and cartilaginous fish (sharks, skates and rays) (Dahn et al., 2007) that have been analyzed, suggesting an evolutionarily conserved mechanism for limb and fin patterning along the A/P axis. Targeted removal of the *ZRS* sequence in mouse (Sagai et al., 2005) leads to limb-specific defects indistinguishable from those in the $Shh^{-/-}$ mouse (Chiang et al., 2001; Kraus et al., 2001) and demonstrates that the *ZRS* provides positive regulatory input for *Shh* expression in the limb.

There is an increasing number of reports of human families, mouse mutants and other mammals with preaxial polydactyly (PPD), or ectopic anterior digits, that have single nucleotide changes scattered throughout the conserved *ZRS* region (Lettice et al., 2002; Lettice et al., 2003; Sagai et al., 2004). In some PPD mouse models, *Shh* is expressed in an ectopic anterior domain, in addition to the posterior domain (Chan, et al., 1995; Masuya et al., 1995; Masuya et al., 1997; Sharpe et al., 1999). Experimentally induced anterior *Shh* expression results in the development of PPD in both chick and mouse (Riddle et al., 1993; Lopez-Martinez et al., 1995; Liu et al., 1998). It is proposed that the *ZRS* integrates regulatory inputs necessary for proper localization of *Shh* expression in the ZPA (Lettice et al., 2002; Lettice et al., 2003; Sagai et al., 2004; Maas and Fallon, 2005; Sagai et al., 2005). The *ZRS* is sufficient to drive reporter gene expression in the ZPA in the mouse limb (e.g., Lettice et al., 2003; Maas and Fallon, 2005). Extensive experiments using PPD-*ZRS*-*LacZ* mouse transgenic reporter assays (Lettice et al., 2008) show that the *ZRS* point mutations in human, mouse and cat drive anterior and posterior limb bud reporter expression in the mouse, similar to *Shh* expression in PPD mutant limb buds. This has been interpreted to mean the wide spread single base pair mutations in the *ZRS* produce a gain of function in the PPD-*ZRS* enhancer sequence (Lettice et al., 2008).

Although there are many reported cases of PPD in animal models and human families, examples of limb-specific *Shh* loss-of-function are rare. Acheiropodia (ACHP) is a recessive human disorder where affected individuals have distally truncated limbs but no other apparent defects in the body (Ianakiev et al., 2001). The mutation for ACHP has been mapped to a deletion of 4–6 kilobases in the *LMBR1* gene, which removes exon 4 and several kilobases of flanking intronic sequence, distinct from the limb-specific *Shh* enhancer in intron 5 (Ianakiev et al., 2001). In the chicken mutant *oligozeugodactyly (ozd), Shh* is never expressed in the developing limbs, but is expressed normally elsewhere in the body, leading to limb-specific defects reminiscent of those found in ACHP (Ros et al., 2003). This phenotype is similar to limbs of the $Shh^{-/-}$ mouse (Chiang et al., 2001; Kraus et al., 2001). In *ozd*, these limb-specific defects include the absence of the ulna and all digits in the wing, and the absence of the fibula and all digits but digit 1 in the foot. However, unlike ACHP and the $Shh^{-/-}$ mouse, the bones that develop in the *ozd* limbs are morphologically normal. We previously showed that the *ozd* mutation is linked to the *LMBR1-Shh* genomic region, and that the open reading frame of *LMBR1* and intronic regions corresponding to the ACHP deletion are normal in the *ozd* mutant (Maas and Fallon, 2004).

Here we report that the spontaneously occurring chicken *ozd* mutant contains a large deletion in *LMBR1*-intron 5, eliminating most of the limb-specific enhancer conserved sequence. In addition, we have developed and utilized a powerful reporter assay designed to analyze enhancer function in the vascularized mesenchyme of the developing limb bud. This assay allows localization of the β-galactosidase (β-gal) activity in the *Shh* domain of the chicken limb bud, representing the first reported enhancer analysis in a vascularized mesenchyme undergoing dynamic growth in the chick embryo. We further extended the potential of this reporter assay, using the *ozd* mutant to show that the early limb bud is prepatterned with *Shh* competence and that a distinct cohort of pre-ZPA cells exists in the early limb bud and this competence does not require *Shh* expression. Lastly, we report that the spontaneously occurring Silkie Breed and *Polydactyly (Po)* chicken PPD mutants have an identical single base pair change within the limb-specific *Shh* enhancer. Introduction of this point mutation into the reporter assay construct is sufficient to drive β-gal activity in both posterior and anterior domains in normal limb buds. This provides strong support for the hypothesis that the nucleotide change is the genetic mechanism for polydactyly in these mutants and also suggests negative regulatory functions for the *ZRS*.

RESULTS

Analysis of the conserved *ZRS* in the chicken *ozd* mutant

Initially we focused our attention on the conserved *ZRS* in the *ozd* chicken mutant because *ozd* mutants lack *Shh* expression specifically in the limb bud (Ros et al., 2003) and the *LMBR1,* intron 3 segregates with the *ozd* phenotype (Maas and Fallon, 2004). We attempted to amplify a small 477 base pair region from the *ZRS* from *ozd* mutants and phenotypically normal chickens from the *ozd* flock, corresponding to the region reported in Sagai et al., 2004. However, PCR-product was not obtained in any *ozd* sample, even using several different primer combinations, indicating that the *ozd* mutation likely had a deletion within the conserved region in *LMBR1,* intron 5. Southern hybridization was performed to determine whether the *ZRS* was present in *ozd* using the 477 base pair sequence as a probe. Although a strong band was detectable from all phenotypically normal samples, this band was not detectable in *ozd* mutant embryos, indicating that most, or all, of this region is deleted in *ozd (*Figure 1A).

To determine the exact sequence that was deleted in *ozd* mutants, we isolated a BAC clone containing the wild type *LMBR1,* intron 5-region by screening high-density replica filters from a chicken BAC library. Primers were designed, 1F to 7R (Figure 1B), within the conserved *ZRS* and used to sequence in both forward and reverse directions (e.g. 1F and 1R, 2F and 2R, and so on, Figure 1B). Sequence analysis of the PCR product obtained in *ozd* mutant samples using these primers showed that the *ozd* mutant contains a deletion of 1654 base pairs within *LMBR1*, intron 5 (Figure 1B) that eliminates all but the first 135 base pairs of the ~800 base pair conserved *ZRS* sequence reported in Lettice et al., 2003, as well as several hundred base pairs of nonconserved intronic sequence (Figure 1D). When we used 5′*ozd* del and 3′*ozd* del primers (Figure 1B), we observed ~3.1kb non-carrier band and ~1.5kb *ozd* carrier band (Figure 1C). The same PCR primers were used to generate both the large (wild type or +/+) and small (*ozd* or −/−) PCR fragment in *ozd* or phenotypically normal (+/+ or +/−) DNA samples. The reaction conditions were identical, but because the small fragment was more robustly amplified than the larger fragment, the small fragment appears brighter than the large fragment (Figure 1C), even though they are present in equimolar amounts; this is not a quantitative assay. Near the 3′ breakpoint (Figure 1D), the *ozd* sequence contains a duplicated octamer that is not present in normal White Leghorn or Brown Leghorn chickens (current carrier and original *ozd* strains, respectively). The sequence does not correspond to a known transcription factor binding-site; the significance of the duplication is unknown.

Development of an *in vivo* reporter assay to analyze *ZRS* function in the chicken limb

To rapidly and accurately study *ZRS* function, we developed an *in vivo* reporter assay using electroporation in the chicken limb field. Although enhancer analysis has been used successfully in the chicken avascular neural tube (Uchikawa et al., 2003), to our knowledge, the results described below represent the first reported enhancer analysis in a chicken vascularized mesenchyme. We cloned a 1.7 kilobase region of *LMBR1,* intron 5 containing the *ZRS* sequence, using genomic DNA from the progeny of Hyline White Leghorn × New Hampshire Red chicken cross (Figure 2A). Although only the core enhancer region is homologous between mouse and chicken, we chose to use the 1.7-kilobase fragment since it correlated with the fragment used successfully in previous mouse reporter analyses (Lettice et al., 2003; Maas and Fallon, 2005). The enhancer fragment was first tested in the *HSP68-lacZ* reporter construct used in mouse reporter gene experiments (Maas and Fallon, 2005). However, in chick, the *HSP68* promoter in this construct was induced upon electroporation, causing widespread β-galactosidase (β-gal) activity at all stages analyzed (n=26/33; Figure 2B-St. 20; Figure 2C- St. 24). Therefore, this construct was used as a control to assess the extent of electroporated limb tissue expected to occur with other constructs in subsequent experiments.

Epstein *et al.* (1999) showed that a 1.1 kilobase fragment of the mouse *Shh* promoter drives reporter gene expression in regions of the central nervous system where *Shh* normally is expressed only when combined with neural specific enhancers. We inserted the 1.7 kilobase chicken sequence containing the *ZRS* into the mouse (m) *SHH* promoter/*lacZ* construct (*ZRS-mShh-lacZ:* Figure 2A). However, β-gal activity was not detected in the chicken limb upon electroporation of this construct.

We hypothesized that species-specific promoters may be necessary for regulatory control analysis in the chick limb bud, and replaced the 1.1 kilobase mouse *Shh* promoter fragment with a 1.1 kilobase region of the chicken (c) *Shh* promoter (ZRS-*cShh-lacZ:* Figure 2A). Embryos electroporated with the *ZRS-cShh-lacZ* construct showed β-gal staining in the ZPA region of the experimental limb through stage 24 (n=43/59; Figures 2E-K). The control *cShh-lacZ* construct, without the *ZRS* enhancer (Figure 2A) did not show β-gal activity (n=9/9, Figure 2D). Taken together these data raise the possibility that, contrary to results in the chick neural tube where the TK promoter was used successfully to drive *Sox2* expression, (Uchikawa et al., 2003), species-specific promoters may be necessary in reporter assays in the chick limb bud vascularized mesenchyme. Our results demonstrate that enhancer analysis can be performed effectively in the vascularized mesenchyme of the chicken limb bud.

A cohort of ZPA-competent cells is present in the *ozd* mutant

Next, we extended our reporter analysis to examine whether β-gal activity could be detected in *ozd* mutant limbs buds, which lack most of the conserved *ZRS* sequence. It has been proposed that the early limb bud is prepatterned to establish a domain of *Shh* competence (Ros et al., 1996; Chiang et al., 2001;te Welscher et al., 2002; Zákány, 2004). The factors necessary for asymmetric *Shh* expression include both activators, such as *HAND2*, *TBX3*, and 5′ *HOXD* genes, and repressors, *GLI3* and *ALX4* (see for example: te Welscher, 2002; Zákány et al., 2004). The activator genes are initially expressed in *ozd* limb buds, but later are down regulated (Ros et al., 2003). Therefore, we reasoned that if these transcription factors, proposed to prepattern the limb bud, act through the *ZRS* to establish a domain of *Shh* expression then β-gal activity would be detectable in this cellular cohort in the *ozd* limb bud. Embryos from the *ozd* flock were electroporated with the *ZRS-cShh-lacZ* reporter construct. Experimental embryos were harvested from St. 19/20 to St. 26 and processed for β-gal staining.

We found that reporter activity is present in posterior *ozd* limb bud mesoderm at all stages analyzed, indicating that *ZRS* sequence is sufficient to initiate β-gal expression in the *ozd* mutant in cells where *Shh* would be expressed in wild type limb buds. At early stages of limb development, when the *ozd* limb is indistinguishable from normal limbs, reporter activity is detectable in the same region in all chick limbs from matings of heterozygote carrier parents, coinciding with the *Shh* expression domain (data not shown). Based on our examination of thousands of *ozd* heterozygous matings (Ros et al., 2003), 25% of these embryos would be homozygous for the *ozd* mutation, but indistinguishable from wild type in the reporter assay used.

At later stages of development when the *ozd* limb begins to exhibit a characteristic *ozd*-shape (Ros et al., 2003), the domain of β-gal activity in *ozd* limbs is noticeably different from that in normal limbs (n=5). Specifically, when compared to normal limbs, the β-gal expression domain in the *ozd* limb bud occupies a greater portion of the developing limb bud along both the anterior-posterior (compare Figure 3A, B with 3D, E) and proximal-distal axes (compare Figures 3C with 3F), but is narrower along the dorsal-ventral axis (compare Figures 3C with 3F). It is possible that these observations relate to the change in size and shape of the *ozd* limb bud beginning at St. 23. The detection of β-gal expression after stage (St.) 23 when *HAND2* mRNA is down-regulated in *ozd* limb buds is likely due to: 1) perdurance of the β-gal protein, prolonging enzymatic activity (Echelard et al.; 1994; 2) a potentially long half-life of HAND2 protein, which at this time is unknown; or 3) the auto-regulatory initiation of *Shh*, which has been reported in both the *Shh* null mouse and chick (Scherz et al., 2007, Sanz-Ezquerro and Tickle, 2000.

The important conclusions from our observations is that detection of β-gal activity in *ozd* limbs demonstrates a cohort of pre-ZPA cells along the emerging limb bud border and that the *ZRS* is required for *Shh* expression in the chicken limb bud in this cellular cohort, establishing the positive regulatory input of the *ZRS* in the chicken model system.

Analysis the *ZRS* in PPD chicken mutants

The Silkie Breed of chicken carries several characteristic, heritable traits that are not linked genetically, including semi-dominant PPD with variable penetrance. The normal (wild type) chicken foot has four toes on each foot, a total of 8-toes. As reported in the classical literature (see Sturkie, 1943; Warren, 1944, for quantitation and more references) Silkie Breed feet most commonly have 5 toes on each foot, (Figure 4A, arrow points to duplicated preaxial digit 1) for a total of 10-toes, but in rare cases Silkie may have between 8 toes total (normal phenotype) and very rarely, 12-toes total. The reason for this variability remains unknown.

Shh expression in Silkie limb buds extended farther distally and anteriorly than in normal limb buds along the posterior sub-apical ectodermal ridge boundary (Figure 4B) this pattern change has also been reported in the PPD mouse mutant *Hemimelic-extra toes* (Blanc et al., 2002). At later stages, posterior *Shh* expression was prolonged and remains more robust in Silkie Breed limbs compared with normal chick limb buds (not shown). *Shh* is also expressed ectopically at the anterior border of the developing limb bud and was first detectable at St. 25+/26 (Figure 4B). Another polydactylous chicken mutant that we studied, *Polydactyly (Po),* had identical PPD and a range of phenotypes similar to those in Silkie, but did not exhibit other inherited traits carried by the Silkie Breed.

First, we cloned the *ZRS* region from the Wisconsin White Leghorn, Brown Leghorn, HiLine, and 003 lines of non-polydactylous chickens to examine genetic variability unrelated to PPD in this genomic region. All lines exhibited occasional polymorphisms within the 1.7 kilobase region, but only the Silkie Breed and *Po* had a single base pair

change within the conserved 800 base pair *ZRS*. This change was identical in Silkie and *Po* (Figure 4C *). We also analyzed genomic DNA samples for the presence of the base pair change from 8- (n=6), 9- (n=1), 10-(n=9), 11- (n=2), and 12- (n=1) toed Silkie chicks and found that all Silkie birds tested were homozygous for this mutation. However, all *Po* mutants that were analyzed (n=4) were heterozygous for the mutation. This reflects the breeding strategies for the two strains of chicken, since the Silkie Breed was always mated with other Silkie Breed birds, while *Po* carriers were outcrossed when the flock was reproduced. It is possible that *Po* originated from an outcross of the Silkie breed and that these identical mutations did not arise independently.

Bouldin and Harfe, 2009 reported that partially penetrant, preaxial polydactyly in the feet of Dorking Breed chickens was accompanied by ectopic anterior apical ectodermal ridge expression of *FGF4*, along with repression of normal cell death in the mesodermal anterior necrotic zone and variable anterior ectopic *Shh* expression; *Shh* was variably expressed. They proposed that PPD in the Dorking Breed was caused by an increase in the autopod cellular progenitor pool brought about by ectopic AER-FGF4 signaling preventing normal cell death. It was proposed further that in the absence of ectopic *Shh* an extra digit 1 would form, whereas in the presence of ectopic *Shh* a digit 2 would form (Bouldin and Harfe, 2009). To explore this further we sequenced (n=4) the Dorking Breed *ZRS*. Interestingly, and distinct from the Silkie Breed and *Po*, we found the conserved *ZRS* region was identical through out its length to the wild type sequence (Figure 4C). We conclude that Dorking Breed and Silkie Breed PPD represent fundamentally different developmental genetic mechanisms for PPD.

While our research was ongoing, (Dorshorst et al., 2010) published data demonstrating the same nucleotide change in the *ZRS* of Silkie and *Po* chickens and the lack of any change in the *ZRS* of the Dorking Breed, all of which we confirm and draw attention to here in the context of the Bouldin and Harfe, (2009) report.

To investigate further the function of the single nucleotide change in the Silkie Breed, we tested a reporter construct similar to that used in the previous *ozd* experiments above, replacing the 1.7 kilobase region from the non-polydactylous White Leghorn chicken with the same region from the Silkie breed (Si-*ZRS-cShh-lacZ*) and assayed at stages 23–24, several stages before anterior ectopic *Shh* is expressed in Silkie limbs. Interestingly, the posterior domain of *Shh* expression is wider in Silkie Breed than in normal limbs at all stages analyzed (compare Figure 3G with Figure 4D). In agreement with this observation, we consistently detected broader posterior and dorsal-ventral domains of β-gal activity in White Leghorn limbs electroporated with the Si-*ZRS-cShh-lacZ* construct. This is similar to the expanded posterior *Shh* expression domain in Silkie Breed compared with limbs electroporated with the wild type construct (compare Figures 3B, C with Figures 4E, F). No anterior β-gal activity was detected at these early stages, possibly indicating a lack necessary conditions for ectopic *Shh* expression. Importantly, at stages when anterior ectopic *Shh* would be expressed in Silkie limbs, normal embryos electroporated with the Si-*ZRS-cShh-lacZ* construct show β-gal expression in both posterior and anterior domains (n= 16/26; Figure 4G). Expression of the control *HSP68-lacZ* construct throughout the limb (Figure 2B and 2C) indicate that the Si-*ZRS-cShh-lacZ* construct is present throughout the entire limb, but necessary conditions in the limb bud only permit reporter gene expression in regions that correlate with *Shh* expression in Silkie limb buds.

DISCUSSION

We report that the chicken *ozd* mutation is caused by a spontaneous deletion of the majority of the highly conserved zone of polarizing activity (ZPA) regulatory sequence (*ZRS*),

supporting the hypothesis that the *ZRS* is necessary to activate *Shh* expression in the posterior of the developing chick limb model system. This is the only documented report of a spontaneous loss of function mutation of the *ZRS*. We developed a novel reporter bioassay to test the function of the enhancer in the developing chicken limb. The results of these experiments establish the chicken limb as an efficient system in which to conduct gene regulatory studies. The *in vivo* reporter assay was extended to show that the *ozd* limb has a cohort of pre-ZPA cells that are prepatterned by trans-acting factors necessary for *Shh* expression in the limb. Lastly, we provide evidence supporting the hypothesis that negative regulatory elements within the enhancer are necessary for normal repression of *Shh* at the anterior of the limb bud.

Positive regulatory function of the long-range limb-specific *Shh* enhancer

Gene-targeted disruption of the *ZRS* enhancer in the mouse leads to a limb phenotype indistinguishable from that of the $Shh^{-/-}$ mouse (Sagai et al., 2005). Thus removal of the *ZRS* leads to a limb-specific loss of *Shh* expression in both mouse and chicken. It is interesting that the *ozd* phenotype is not the same as the mouse enhancer knockout phenotype, even though both deletions remove a functional enhancer. A main difference is in the stylopod-zeugopod interface where the mouse shows the initiation of the tibia and fibula, which are severely truncated, but their interaction with the femur shows a rudimentary knee joint. The *ozd* leg shows a recognizable tibia with normal indication of a knee joint, but the absence of the fibula. In the forelimb zeugopod, the mouse develops one long bone fused to the humerus, while the chick forms an independent long bone recognizable as the radius and a normal relationship with the proximal humerus. These phenotypic differences may represent a fundamental difference in the effect of the loss of *Shh* expression between mouse and chicken limb development; and/or may be a fundamental difference in morphogenesis, reflecting mammalian versus reptile-bird lineages, which were separated 300 million years ago. An unlikely alternative is that the sequence within the first 135 base pairs, which is present in *ozd* but not in the mouse knockout, is sufficient for controlling parallel, *Shh* independent morphogenetic functions during skeletal patterning.

The precise control of ZPA-restricted *Shh* expression may require regulatory inputs from regions outside of the 800 base pair limb-specific enhancer. Sagai et al., 2005 identified two additional blocks of homology within the *LMBR1* locus that are highly conserved between teleost fish and mammals. Although these homologous sequences are located in regions other than intron 5, the ordering and spacing of the elements is conserved. As already noted, human patients affected with ACHP have a phenotype similar to *ozd* mutants, but show a unique, very large deletion in the region of intron 3 and associated coding region of the *LMBR1* gene that is outside of the limb-specific *Shh* enhancer. Although the ACHP deletion removes part of the coding region of *LMBR1*, we previously showed that the LMBR1 protein is not required for normal chick limb development (Maas and Fallon, 2004). Sequence analysis has revealed no apparent regions of homology between other species within the ACHP deletion, and it has been proposed that the ACHP deletion eliminates the human-specific *Shh* limb enhancer (Sagai, 2005). However, to our knowledge, the *ZRS* within *LMBR1,* intron 5 has not been sequenced in ACHP and it is possible that additional regions of *LMBR1* are mutated in ACHP. It is also possible that the ACHP deletion could affect *Shh* expression in the limb without removing the limb-specific *Shh* enhancer. Specifically, the very large ACHP deletion could alter spacing in the *LMBR1* region of the chromosome, causing a steric limitation that interferes with *ZRS* physical contact with the *Shh* locus and or prevents the chromosomal looping associated with initiating *Shh* transcription (Amano et al., 2009). In this elegant study in the mouse, the authors demonstrate that chromosome contact in posterior limb bud border cells continues in the

Δ*ZRS* mouse. This indicates that the *ZRS* itself does not control this contact, which the authors propose must be controlled by other determinants (Amano et al., 2009); these determinants could be in *LMBR1,* intron 3 and associated coding region of the ACHP deletion. Either one of these factors could prevent normal *ZRS* function, or necessary interaction between the *ZRS* and the *Shh* promoter, precluding activation of limb specific *Shh* in the ACHP limb buds.

Does the absence of the limb-specific enhancer in *ozd* prevent prepatterning of the prospective ZPA cellular cohort?

Uncovering the genetic mechanism of *Shh* expression in the limb has allowed closer examination of multiple facets of gene regulation in limb development. The transcription factors *HAND2* and the 5-prime *HOXD* genes are expressed along the emerging limb bud posterior border and both are required for *Shh* expression (Charite et al., 2000; Fernandez-Teran et al. 2000; Zákány et al., 2004). In parallel, loss of function studies indicate that HAND2 is required to exclude the expression of the repressors *GLI3* and *ALX4* from the posterior limb bud border, restricting them to the anterior limb mesenchyme (te Welscher et al., 2002). A model has been proposed (te Welscher et al., 2002) that the mutually exclusive expression of anterior *GLI3* and posterior *HAND2* at limb bud emergence represents a prepattern of gene expression that result in the posterior asymmetric expression of *Shh*. Follow-up studies have demonstrated strong support for this hypothesis, in that co-immuno-precipitation of HAND2 shows interaction with HOXD13. Also, HAND2 binding to the *ZRS* was demonstrated with chromatin immunoprecipitation, (Galli et al., 2010). Further support for a prepattern is that the *Shh* null mouse (Chiang et al., 2001; Kraus, 2001) and the chick *ozd* mutant (Ros et al., 2003) show initial posterior limb bud expression of *HAND2* and *HOXD13*, which subsequently fails to reach the higher levels of expression and extensive distribution that develops with the presence of *Shh*. Importantly for our study and conclusions is the fact that all six of the HAND2 binding sites in the *ZRS* (Galli et al., 2010), are eliminated by the *ozd ZRS* mutation.

With the reporter assay described here, detection of β-gal activity in the *ozd* limbs that lack the enhancer and never express *Shh*, supports the hypothesis that the early limb bud contains a cohort cells expressing *HAND2* that are normally destined to become the ZPA. *Shh* expression is not necessary for initiating HAND2 expression. Our data indicate that the *ozd* phenotype probably is caused solely by the loss of the enhancer region, which is necessary for limb *Shh* expression. Nevertheless, the emerging *ozd* early limb bud is prepatterned to establish a domain of *Shh* competence. This supports the hypothesis that the activator transcription factors necessary for *Shh* expression in the limb act through the *ZRS* conserved sequence and are stabilized by SHH. In addition, our reporter assay represents an important advance for the study of regulatory elements in the chicken, since transgenic strategies, a common mode of enhancer analysis in other systems, are not possible in the chicken. Additionally, our reporter assay could be readily adapted for the study of other regulatory elements necessary for limb development.

Are negative *ZRS* functions disrupted in PPD mutants?

In the early limb bud, posterior *Shh* expression is noticeably expanded in PPD limb buds compared to nonpolydactylous limb buds. It is only much later in limb development that ectopic *Shh* expression becomes detectable at the anterior of the limb bud of PPD mutants. At the same time posterior *Shh* expression persists for a longer time in PPD limb buds than in normal limb buds. These observations demonstrate that the point mutations, allowing *Shh* expression at the anterior border, also increases the extent of normal posterior *Shh* expression.

Amano et al., (2009) propose that there are three chromosome conformations associated with the *ZRS* in the limb bud mesoderm. The first is an active conformation, found in the ZPA region, where the *ZRS* is physically in contact with the *Shh* start site, followed by chromosome looping and initiation of *Shh* transcription. The second is a silent conformation, found in the middle of the limb bud where the *ZRS* is far from the *Shh* locus and *Shh* transcription has not been observed in this region. The third is a poised conformation, found in the limb bud anterior border mesoderm where physical interaction of the *ZRS* with the *Shh* locus is observed, but looping is not observed and transcription of *Shh* does not occur. Because the *ZRS* interacts with the *Shh* start site in anterior mesoderm, it is possible that the single base pair changes in the *ZRS* associated with PPD increase the affinity of the *ZRS* for the *Shh* start site and *Shh* transcription is initiated. Why this would occur only late in limb development and not parallel with the posterior *ZPA Shh* expression, needs to be evaluated.

Because the same genes are probably required for *Shh* activation in both posterior and anterior locations, one possibility for the delayed anterior expression is that it takes longer to establish a domain of *Shh* competence in the anterior limb in PPD mutants, since genes required for *Shh* expression are normally repressed in this location. In this context the possibility must be explored that the factors that repress *Shh* expression in limb cells other than the ZPA, also require the *ZRS* for function. Recent studies show that loss of ETV4 and ETV5 function and reduction of TWIST1 function result in anterior limb bud *Shh* expression and subsequent PPD (Firulli et al., 2005; Firulli et al., 2007; Zhang et al., 2009; Mao et al., 2009; Zhang et al., 2010; Krawchuk et al., 2010). These genes are required for posterior restriction of *Shh*. It is proposed that a balance at least of TWIST1 levels with HAND2 levels of expression from anterior to posterior, results in restriction of *Shh* to the posterior border and its repression anteriorly. While the interaction of the *ZRS* with HAND2 to initiate and maintain ZPA-*Shh* expression is established (Galli et al., 2010), it remains to be explored whether the *ZRS* plays a second role in the function of different factors such as TWIST1 or ETV4/5 that repress *Shh* expression in the rest of the limb bud mesoderm. The observation of the anterior poised ZRS-bearing chromosome configuration in anterior limb mesoderm (Amano et al., 2009) should be integral to approaches designed to understand the mechanisms that allow anterior *Shh* expression leading to PPD.

EXPERIMENTAL PROCEDURES

Chicken strains and embryos

White Leghorn, Brown Leghorn, Hyline, Silkie Breed, and *oligozeugodactyly* (*ozd*) embryos were obtained from mating flocks at the University of Wisconsin Poultry Research Lab (Madison, WI). *Polydactyly (Po)* and normal carrier strain 003 eggs were obtained from UC-Davis. Eggs were incubated, opened, and staged as described (Hamburger and Hamilton, 1951; Ros et al., 2000). Dorking chicken blood was obtained from the Pat Handrick Farm, Belleville WI.

Southern blot analysis

Genomic DNA was prepared from St. 26 normal or *ozd* mutant chicken embryos. 10 μg of each sample was digested overnight, electrophoresed, and transferred by capillary action onto uncharged nylon membranes. Membranes were hybridized with 1×10^6 cpm denatured 477 base pair *ZRS* probe and hybridization and washing was performed according to standard procedure.

Identification of a chicken BAC clone containing *Lmbr1* intron 5

A High Density Replica Filter Library of chicken BAC clones (CHORI-Oakland, CA) was prehybridized for 1 hour at 65°C in Church's buffer and then hybridized with the chicken *ZRS* sequence and a control probe from *C. briggsae* (to locate "anchor spots" on the developed autoradiograph) at 65°C overnight. Filters were washed with Church's wash several times at 65°C and exposed to film. Positive BAC clones were grown in LB medium containing 20-μg/ml chloramphenicol.

BAC sequencing and identification of *ozd* mutation

BAC DNA was purified using the Qiagen Large Construct Purification Kit and sequenced with intron 5-1F (5′-CACAGAGTCTGTGGATTAAGAGG-3′) and intron 5-7R (5′-CACTCATTTCCAACAATTTATGG-3′) primers (1 μg DNA per reaction). Bands were amplified from both wild type and *ozd* genomic DNA samples (fragments of 3.1 and 1.5 kilobases, respectively) using 5′ *ozd* del (5′-CCACTGCTAACAGAGTACCTTGG-3′) and 3′ *ozd* del (5′-GATAGCACAGAGATTGGTATTCC-3′) primers.

Generation of chicken 1.7 kilobase fragment reporter constructs

The mouse 1.7 kb fragment containing the 800 base pair *ZRS* was aligned with the chicken genome. ~1000 base pairs in the center were homologous and ~300 base pairs on either side of the homologous region were included so chicken and mouse constructs were of similar size. 1.7 kilobase fragments were PCR amplified with primers containing NotI (5′) or BamHI (3′) restriction sites from both normal White Leghorn and Silkie breed samples (primers used: 5′ NotI primer- 5′-ATAAGAATGCGGCCGCGACACTGGAATAGCCTGAAG- 3′; 3′ BamHI primer- 5′-CGGGATCCCAGGGAATTTCAGTACTGG- 3′). The fragments were cloned into a pBR322 construct upstream of a 1.1 kilobase chicken *Shh* promoter fragment (primers- 5′ BamHI primer-5′-CGGGATCCGCCATTCACTTTGCCGGGAT- 3′; 3′ SpeI primer- 5′-GGACTAGTCCAATTACTTCACAGCTCTCTGTG- 3′).

Electroporation experiments for reporter gene analysis in chicken

DNA was purified using Qiafilter maxiprep kits (Qiagen) and resuspended at a concentration of ~4–5 μg/μl in TE, pH 8.0. We followed the protocol for limb-field electroporation described in Krull, 2004. Briefly, DNA solution was injected into the presumptive right hind limb region of stage (St.) 13–14 embryos using a glass pipette followed by several drops of chicken Ringer's solution. 0.5 mm, electrodes consisted of a 0.5mm straight platinum wire cathode held over the top of the hind limb-forming region and a "hockey stick" anode (0.5mm platinum wire bent at a 90 degree angle) under the hind limb-forming region containing the injected DNA solution. Injected limbs were electroporated with 3 pulses of 13V, 50 ms pulse length and 100 ms interval length using a BTX, ECM 830 electroporator. Embryos were harvested 1–3 days after electroporation for visualization of reporter gene expression.

β-galactosidase staining of embryos

Embryos were harvested into ice cold phosphate buffered saline (PBS) and fixed in 2% formaldehyde, 0.2% glutaraldehyde, 0.2% NP-40 in PBS for 1–1.5 hours at 4°, washed 3 times in PBS, and incubated in 5mM $K^{++}FeCN$, 5mM $KFe^{+++}CN$, 0.2% NP-40, 2 mM MgCl2, 0.5 mg/ml XGAL in PBS at 37°C overnight. Embryos were washed in PBS and stored in PBS+ 10mM EDTA.

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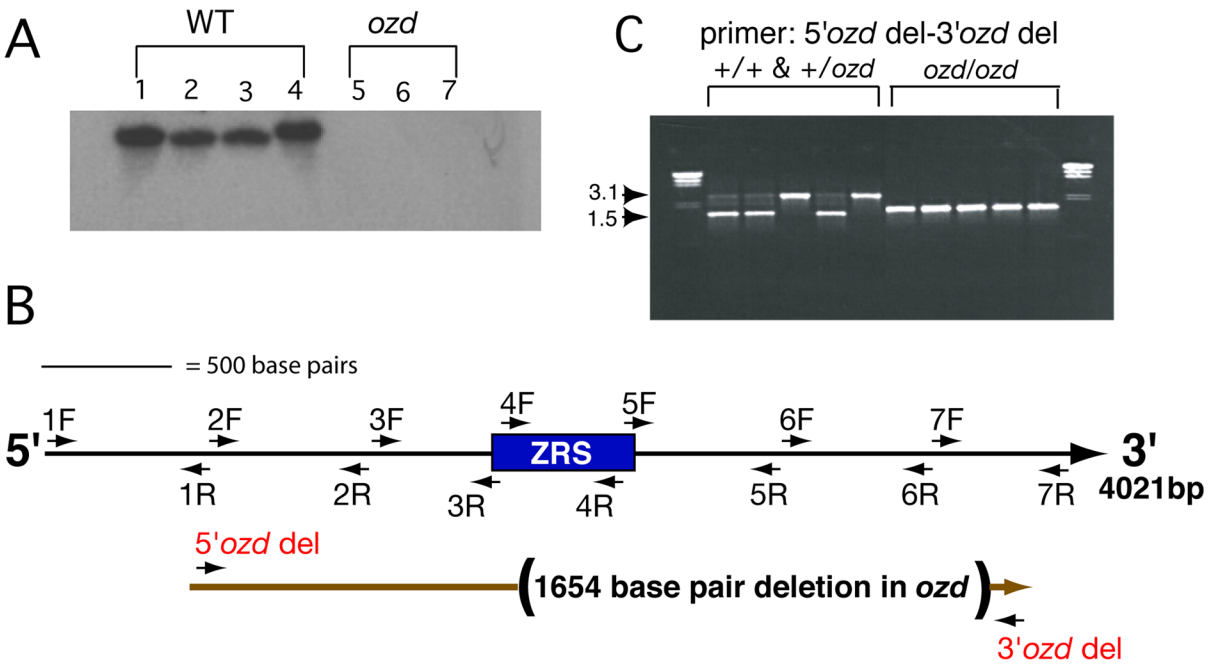

D

5' end of *ozd* deletion

```
WT  5'CACACACACAGAGTCTGTGGATTAAGAGGTTAACTCCTGGAACATCAA 3'
ozd  CACACACACAGAGTCTGTGGATT
```

3' end of *ozd* deletion

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WT  5'GATAAATTCAGAAA--------ACTCACTGGGTTGAATTCAAGACAAT 3'
ozd  GATAAATTCAGAAATTCAGAAAACTCACTGGGTTG
```

Figure 1. The chicken *oligozeugodactyly (ozd)* mutant contains a deletion of the *ZRS*

(A) Genomic DNA from phenotypically normal (normal, +/+, and carriers, +/*ozd*, lanes 1–4) or known *ozd* mutants (lanes 5–7) was digested with SalI and BamHI. The resulting blot was probed with a 477 base pair *ZRS* fragment. A distinct band is present in all of the phenotypically normal lanes, but not in any *ozd* lane. (B) A BAC clone containing the conserved 477 base pair intron 5 region (rectangle) was obtained and ~4 kilobases of intron 5 was sequenced. Approximate locations of primers are shown as arrows, where the direction of the arrowhead depicts whether the primer was used to sequence (forward primers- arrows facing away from conserved region) or to amplify a PCR product from *ozd* samples (reverse primers- arrows facing towards conserved region). Primer pairs are numbered. The mutation in *ozd* was determined to be a 1654 base pair deletion within *LMBR1* intron 5, deleting all but the first 135 base pairs of the *ZRS* and several hundred base pairs of intronic sequence (shown in parentheses). (C) 5′ *ozd* del and 3′ *ozd* del primers were used to amplify fragments from normal, heterozygous, and *ozd* genomic DNA samples. The normal band is ~3.1 kilobases and the mutant band is ~1.5 kilobases, for further explanation, see Results section of text. (D) Shown here are the 5′ and 3′ breakpoints in *ozd* mutants. Note the duplicated octamer (shown in red) near the 3′ breakpoint of the *ozd* deletion.

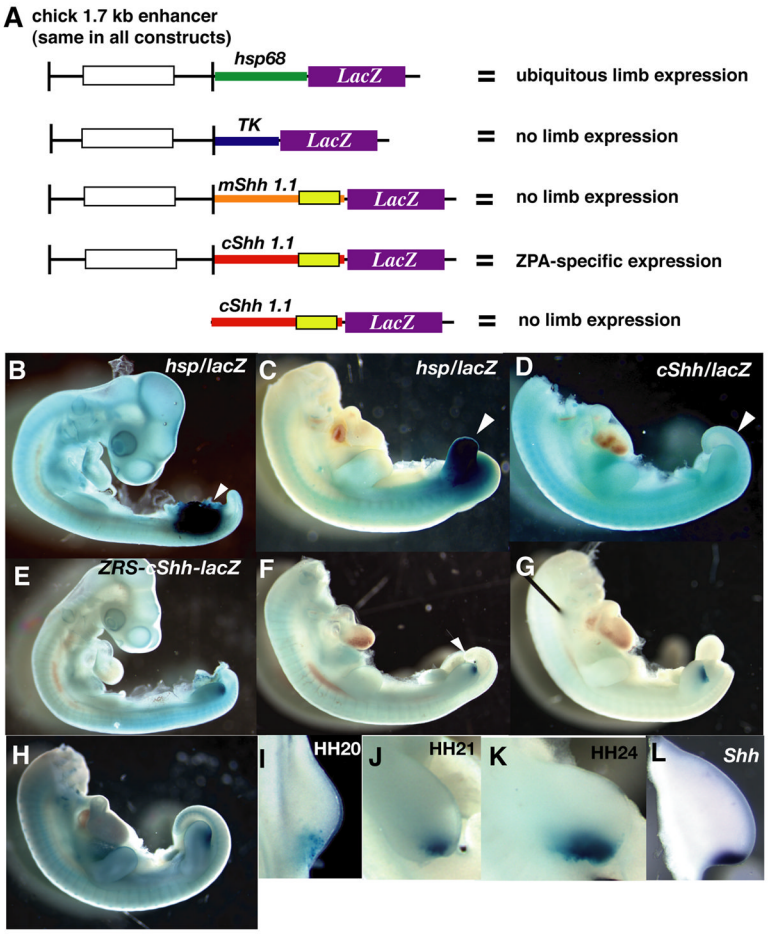

Figure 2. An *in vivo* reporter assay analyzing the *ZRS* function in the chicken limb drives reporter gene expression in the ZPA

(A) A summary of constructs tested in reporter assay. Reporter constructs containing a 1.7 kilobase fragment containing the conserved *ZRS* (conserved 800 base pair region represented by white rectangle) upstream of either the *HSP68* (~800 base pairs), thymidine kinase (TK- ~500 base pairs), mouse minimal *Shh (*~1.1 kilobase), or chicken minimal *Shh (*~1.1 kilobase) promoters and *lacZ,* as well as a control construct containing the chicken minimal *Shh* promoter and *lacZ* but no enhancer sequence, were tested in an *in vivo* reporter assay in the chicken. The yellow rectangle in the *mShh* and *cShh* promoters represents a region of high homology in both promoters. Of these constructs, only the ZRS-*cShh-lacZ* construct drives reporter gene expression in the ZPA. (B, C) The *HSP68-lacZ construct* reproducibly drives *lacZ* expression throughout the entire limb bud at all stages analyzed (arrowheads point to experimental limb), including early (B, St. 20) and later (C, St. 24) stages. (D) No β-galactosidase was detectable in limbs electroporated with a control *cShh-lacZ* construct lacking the *ZRS* (n=9/9). (E-K) A high percentage of embryos electroporated with the *ZRS-cShh-lacZ* construct showed β-gal activity in the limb (n=43/59), and in all positively staining embryos, β-gal activity is restricted to the posterior limb in the endogenous *Shh* ZPA expression domain at all stages (St.) analyzed, including St. 20 (E, I), St. 21 (F, J), St. 22 (G), St. 26 (H, K). Also shown is *Shh* mRNA expression for comparison (L). In I-L, posterior is on the bottom.

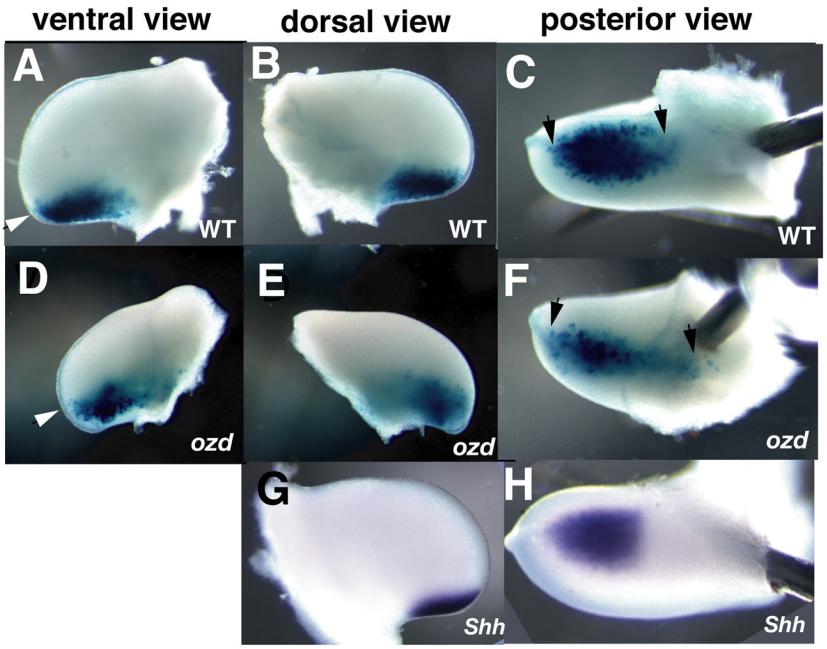

Figure 3. Expression of the reporter gene in *ozd* mutant limbs indicates the presence of a cohort of ZPA precursor cells in the early limb

Limbs at stage 23–24 were harvested from equivalently staged normal (A, B, C) or *ozd* (D, E, F) embryos electroporated with the *ZRS-cShh-lacZ* construct. The same limb is shown from ventral (A, D), dorsal (B, E), or posterior (C, F) views to visualize the extent of β-gal activity. In posterior views, ventral is to the top and dorsal is to the bottom. *Shh* mRNA staining in normal limb buds is also shown (G, H) for reference. Note the expanded β-gal staining domain in the *ozd* limb buds (indicated by white and black arrowheads). Scale bars are included for comparison between samples. St refers to Hamburger and Hamilton 1951, stages of chick development.

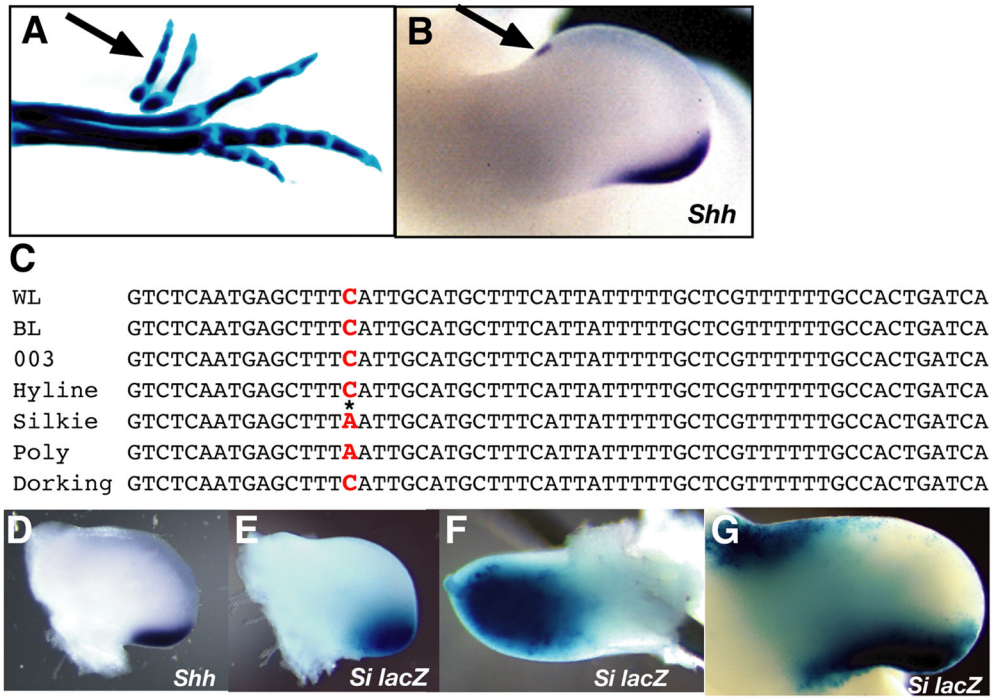

Figure 4. Identification of a unique single base pair change in the *ZRS* of the Silkie and Polydactyly chicken PPD mutants

(A) Day E10 cartilage stained Silkie foot with preaxial extra digit 1 (arrow-top). (B) St. 26 Silkie Breed limb demonstrating ectopic anterior *Shh* mRNA expression (arrow-top), in addition to broadened posterior *Shh* expression in the ZPA (bottom). (C) The normal conserved *ZRS* sequence in the nonpolydactylous chicken strains: White Leghorn (WL), Brown Leghorn (BL), strain 003 and Hyline (HL). An identical base pair change C to A within the *ZRS* region (red) denoted by an asterisk, was found in the PPD Silkie and *Polydactyly (Po)* chicken strains. The PPD Dorking strain was found to have a normal sequence throughout the ZRS. (D) Posterior *Shh* expression was expanded distally and anteriorly in Silkie limb buds at all stages analyzed (shown here, St. 23–24). (E-G)β-galactosidase staining in wild type limbs electroporated with a reporter construct containing the *ZRS* sequence from Silkie chickens [Si-*ZRS-cShh-lacZ* (*Si lacZ*)]. (E, F) St 23–24 showed no detectable anterior staining, but posterior β-gal activity was expanded similar to the expansion of *Shh* expression in the Silkie limb at St 23–24. (G) St. 25+ limb showed both ectopic anterior and expanded posterior β-gal activity.