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Progressive Loss of Function in a Limb Enhancer during Snake Evolution

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SUMMARY

The evolution of body shape is thought to be tightly coupled to changes in regulatory sequences, but specific molecular events associated with major morphological transitions in vertebrates have remained elusive. We identified snake-specific sequence changes within an otherwise highly conserved long-range limb enhancer of *Sonic hedgehog* (*Shh*). Transgenic mouse reporter assays revealed that the in vivo activity pattern of the enhancer is conserved across a wide range of vertebrates, including fish, but not in snakes. Genomic substitution of the mouse enhancer with its human or fish ortholog results in normal limb development. In contrast, replacement with snake orthologs caused severe limb reduction. Synthetic restoration of a single transcription factor binding site lost in the snake lineage reinstated full in vivo function to the snake enhancer. Our results demonstrate changes in a regulatory sequence associated with a major body plan transition and highlight the role of enhancers in morphological evolution.

INTRODUCTION

Distant-acting transcriptional enhancers are a major class of tissue-specific regulatory DNA sequences that has been implicated in morphological evolution in vertebrates (Chan et al., 2010; Cooper et al., 2014; Cretekos et al., 2008; Guenther et al., 2014; Guerreiro et al., 2013; Indjeian et al., 2016; Jones et al., 2012; Lopez-Rios et al., 2014; McLean et al., 2011; Prabhakar et al., 2008). Sequence changes in non-coding regulatory DNA are hypothesized to be a main driver of changes in body shape (Britten and Davidson, 1969; Carroll, 2008; King and Wilson, 1975; Wray, 2007), but many aspects of this complex interplay between molecular changes in regulatory sequences and morphological adaptations across the vertebrate tree remain the subject of considerable debate (Hoekstra, 2012; Wittkopp and Kalay, 2011; Wray, 2007).

In the present study, we utilized a series of recently sequenced snake genomes to study the molecular and functional evolution of a critical limb enhancer in snakes and examine its possible role in limb loss. Our analysis focuses on one of the best-studied vertebrate enhancers, the Zone of Polarizing Activity [ZPA] Regulatory Sequence (ZRS, also known as MFCS1) (Lettice et al., 2003, 2008, 2012, 2014; Sagai et al., 2004, 2005; Zeller and Zuniga, 2007). The ZRS is a limb-specific enhancer of the *Sonic hedgehog* (*Shh*) gene that is located at the extreme distance of nearly one million base pairs from its target promoter. During limb development, the enhancer is active in the posterior limb bud mesenchyme (Figure 1A), where its activity is critically required for normal limb development in mouse (Sagai et al., 2005). Single-nucleotide mutations within the ZRS cause limb malformations, such as preaxial polydactyly, in multiple vertebrate species including humans (Hill and Lettice, 2013; Lettice et al., 2003, 2008; VanderMeer and Ahituv, 2011). Surprisingly, we observed that the sequence of this limb enhancer is conserved throughout nearly all examined species in the snake lineage. In basal snakes, which retain vestigial limbs, it is highly conserved, whereas it underwent a rapid increase in substitution rate in advanced snakes, in which all skeletal limb structures have disappeared. Consistent with this, we provide evidence that the snake enhancer progressively lost its in vivo function as the body plan evolved from basal to advanced snakes. Finally, we identify a specific subset of nucleotide changes within the enhancer that contribute to its functional degeneration in snakes and show in a mouse model that synthetic reintroduction of just one degraded transcription factor binding site is sufficient to recreate the ancestral function and to rescue normal limb formation in vivo.

RESULTS

A Critical Limb Enhancer Is Evolutionarily Conserved but Highly Diverged in Snakes

To explore the potential role of the ZRS limb enhancer in snake evolution, we examined the draft genomes of six snake species

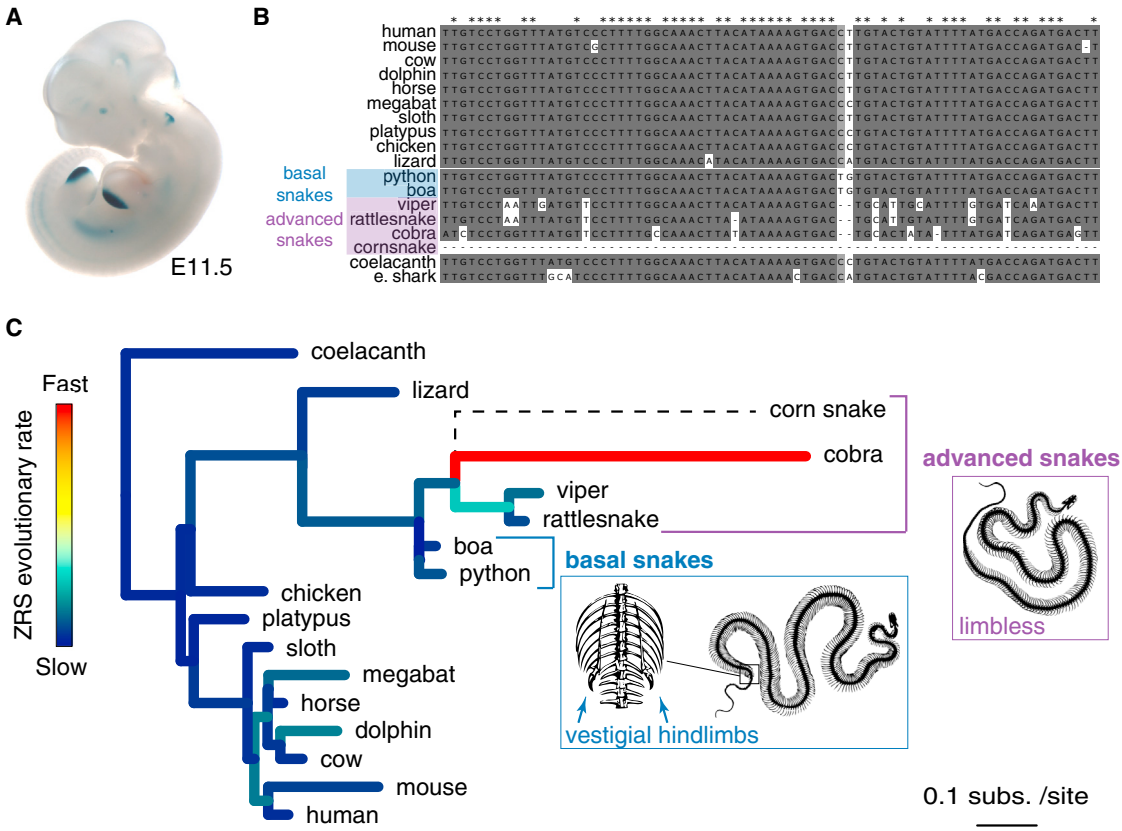

Figure 1. Evolution of a Limb Enhancer across the Vertebrate Tree

(A) Human ZRS enhancer activity in a mid-gestation (E11.5) mouse embryo. Staining in structures other than limb was not reproducible in additional transgenic embryos and due to ectopic effects.

(B) Comparison of the core ZRS region across 18 different vertebrate species including two basal (blue) and four advanced (purple) snakes. See Figure S1 for full alignment.

(C) Phylogeny of vertebrate species used in the study (based on UCSC [https://genome.ucsc.edu/cgi-bin/hgGateway] and Hsiang et al., 2015; Pyron et al., 2013). Branch length indicates absolute ZRS substitution rate, colors indicate relative ZRS evolutionary rate compared to other embryonic enhancers (see Figure S2 and Method Details). The schematic illustrations of snake skeletons were drawn using images from Romanes (1892), http://www.zoochat.com/, and http://www.skullcleaning.com/ as templates.

See also Figures S1 and S2.

including the Burmese python (*Python molurus bivittatus*) (Castoe et al., 2013), boa constrictor (*Boa constrictor constrictor*), king cobra (*Ophiophagus hannah*) (Vonk et al., 2013), speckled rattlesnake (*Crotalus mitchellii pyrrhus*), viper (*Vipera berus berus*), and corn snake (*Pantherophis guttatus*) (Ullate-Agote et al., 2014). These species represent different morphological stages within the evolutionary history of snakes (Apesteguía and Zaher, 2006; Martill et al., 2015), from basal snakes (boa and python) that retained a vestigial pelvic girdle and rudimentary hindlimbs, to advanced snakes (viper, rattlesnake, king cobra, and corn snake) that completely lost all skeletal limb structures and represent the majority (>85%) of all extant snake species (Lawson et al., 2005; Pyron et al., 2013). Nearly all of the snake species studied have a ZRS-orthologous sequence (Figures 1B and S1). However, while the ZRS enhancer of basal snakes shares ~80% nucleotide identity with the orthologous region from limbed lizards and shows a substitution rate similar to other vertebrate ZRS orthologs, the ZRS of advanced snakes displays a substantially increased number of substitutions compared to other enhancers ($p = 0.012$, permutation test; Figures 1B, 1C, and S2; Table S4). This fast evolutionary rate clearly distinguishes the ZRS from other limb enhancers, which do not show such an increase in substitutions (Figure S2) (Infante et al., 2015). Thus, while nearly all snake species examined have a ZRS enhancer, a loss of evolutionary constraint on this enhancer coincides with the complete loss of limb structures at the transition from basal to advanced snakes.

Loss of Region-Specific Limb Enhancer Activity in Snakes

To systematically examine whether the sequence changes observed in different snake ZRS orthologs alter the in vivo function of the enhancer, we used a transgenic mouse enhancer reporter assay (http://enhancer.lbl.gov/) (Kothary et al., 1989; Visel et al., 2007). We determined ZRS enhancer activity patterns for 16 different species covering a wide range of jawed vertebrates,

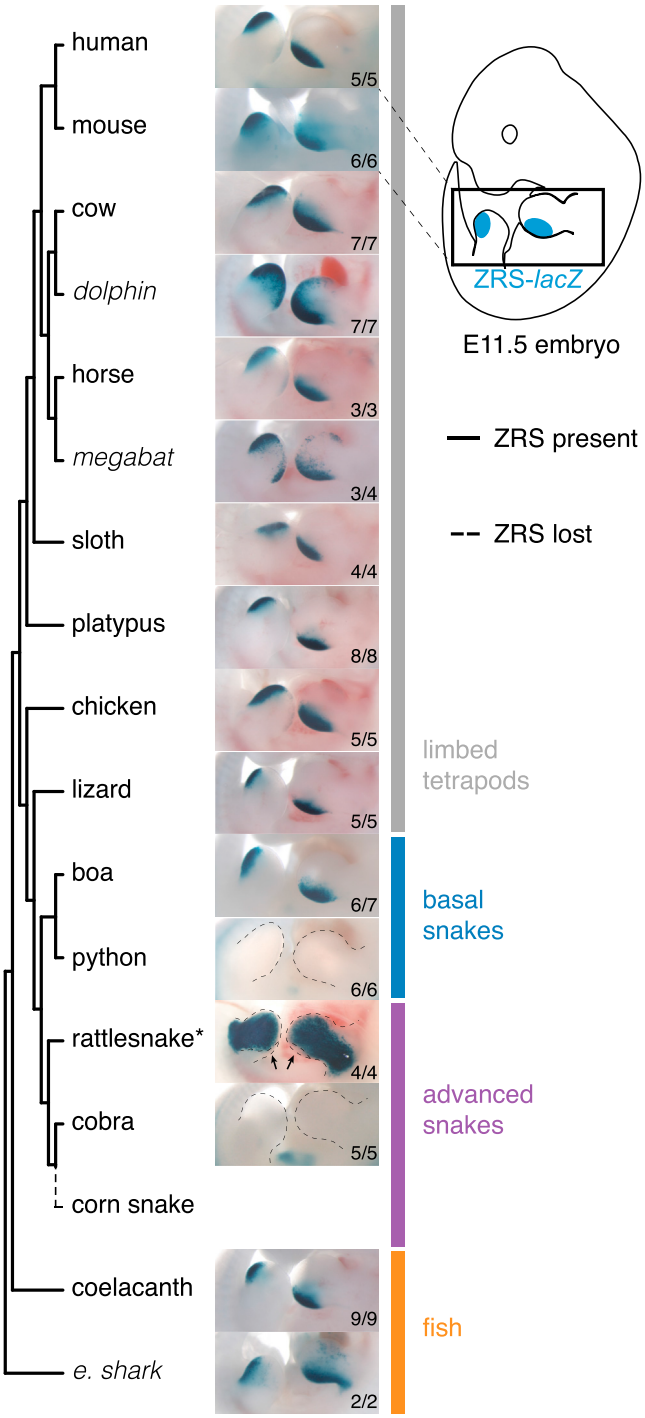

Figure 2. Comparison of Enhancer Activity across Jawed Vertebrates
Enhancer activities for 16 different vertebrate species in the limb buds of transgenic E11.5 stage mouse embryos. Numbers of embryos with lacZ activity in the limb over the total number of transgenic embryos screened are indicated. Some species (marked in italics) were active in the ZPA of the limb buds but had additional activity expanded anteriorly (dolphin and megabat) or proximally (elephant shark). *The rattlesnake ZRS enhancer drives an ectopic reporter activity pattern that does not include the ZPA (arrows point to the ZPA area without detectable LacZ activity).

including cartilaginous and bony fishes (elephant shark and coelacanth), four snakes (boa, python, rattlesnake, and cobra), and ten limbed tetrapods at mid-gestation (embryonic day [E] 11.5), a time point when the mouse ZRS is active (Figures 1A and 2) (Lettice et al., 2003). The orthologs from nine finned or limbed vertebrates (coelacanth, lizard, chicken, platypus, sloth, horse, cow, mouse, and human) displayed reproducible patterns of activity in the posterior limb bud that were indistinguishable from the activity of the mouse enhancer (Figure 2), confirming the deep conservation of its function across vertebrates with paired appendages (Dahn et al., 2007; Lettice et al., 2003; Sagai et al., 2004). ZRS orthologs from three species were active in the ZPA of mouse limb buds but also had activity expanded anteriorly (dolphin and megabat) or proximally (elephant shark). In contrast, in four out of five basal and advanced snake species examined, either the enhancer activity in the ZPA or the enhancer sequence itself was lost (Figure 2). Among them, the rattlesnake ZRS displayed an ectopic limb activity pattern that did not include the ZPA and may be related to an ~180-bp insertion specifically found in the viper and rattlesnake lineage (Figures 2 and S1). Only the ZRS of boa, which diverged from python 63–96 million years ago (Esquerré and Scott Keogh, 2016) and among the examined snakes is the one showing the lowest nucleotide substitution rate with respect to that of the lizard, retained activity in the ZPA. Notably, the ZRS from all advanced snakes examined (rattlesnake and cobra) completely lost ZPA-specific activity.

Snake Enhancer Knockin Causes Severe Limb Truncation in Mice

To assess the extent to which the observed activity changes in transgenic reporter assays affect vertebrate limb morphology in vivo, we employed CRISPR/Cas9 genome editing to generate a series of knockin (KI) mice where the functionally critical 1.3-kb core region of the ZRS (Figure S3) was replaced with the orthologous sequences of the same length from other species. We first replaced the mouse ZRS with the orthologs from human (73% sequence identity to the mouse ZRS) and coelacanth (57% sequence identity to the human ZRS), whose last common ancestor lived approximately 400 million years ago. Both the human and coelacanth orthologs resulted in *Shh* expression at the onset of limb bud formation that was indistinguishable from wild-type and rescued the formation of fully developed limbs (Figures 3 and S4G–S4J), indicating that despite considerable evolutionary distance between mammals and fish, the enhancers of mouse, human, and coelacanth are largely functionally interchangeable. In contrast, replacing the mouse ZRS with the orthologous cobra sequence resulted in a complete loss of *Shh* expression and a truncated limb phenotype, affecting both the fore- and hindlimbs, that is indistinguishable from the phenotype caused by deletion of the mouse enhancer (Figures 3, S3, and S4G) (Sagai et al., 2005). This result confirms that despite recognizable sequence conservation, the cobra sequence lacks limb enhancer function and is therefore unable to support limb development. The less diverged python ZRS resulted in a similar but a slightly milder phenotype. While most skeletal forelimb and hindlimb elements distal of the stylopod:zeugopod junction were also severely affected, the python ZRS resulted in formation of

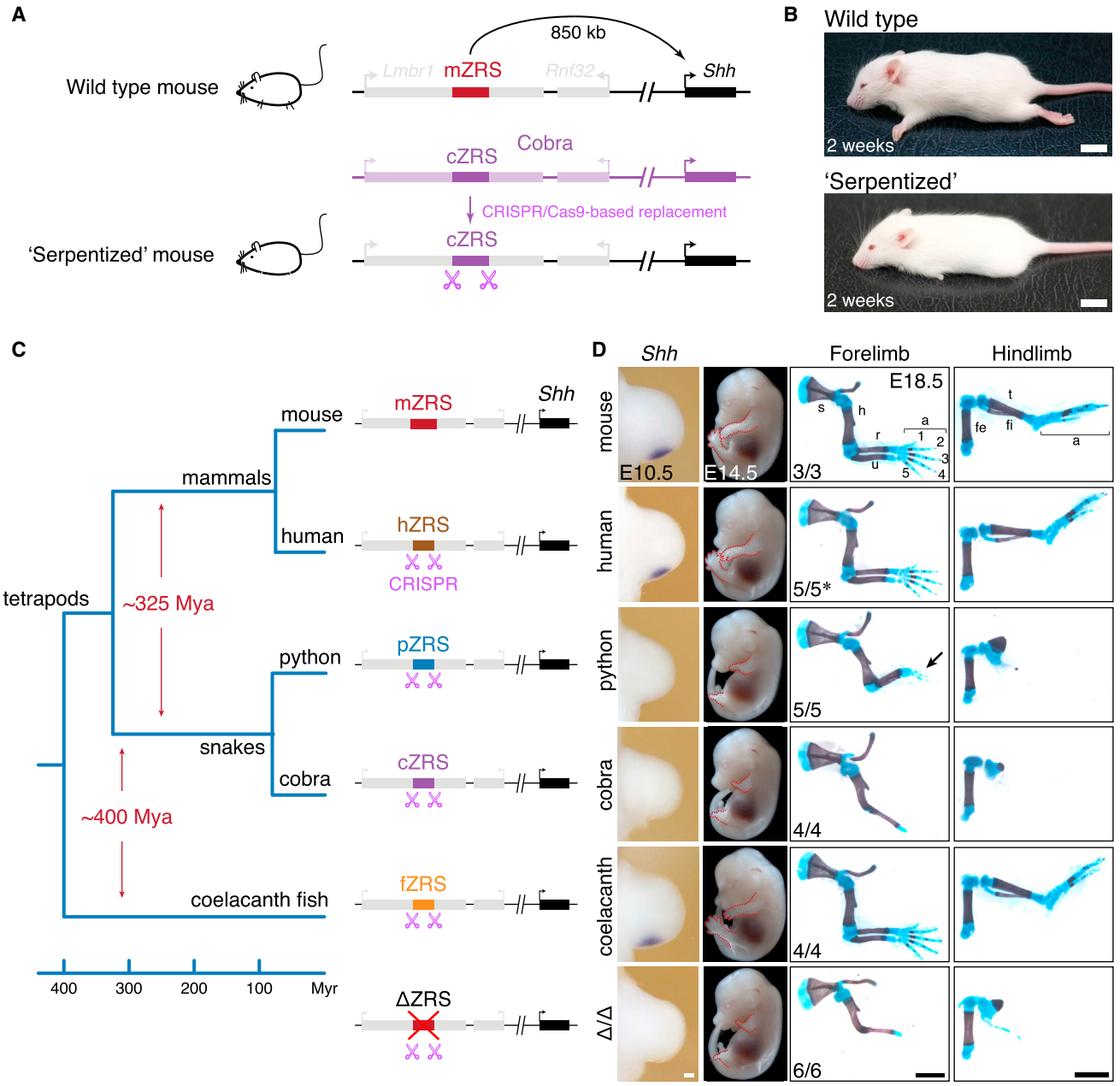

Figure 3. Limb Phenotypes of Knockin Mice with ZRS Orthologs from Other Vertebrate Species

(A) CRISPR/Cas9-mediated replacement of the mouse ZRS sequence with an orthologous sequence from cobra. Schematic of the mouse *Shh* locus is shown at the top. The ZRS is located in the intron of the *Lmbr1* gene (intron-exon structure not shown), 850 kb away from the promoter of *Shh*. A homologous locus from king cobra with the cobra ZRS enhancer (cZRS) is indicated in purple. A CRISPR/Cas9-modified "serpentized" mouse *Shh* locus is shown below. See also Figures S4A–S4F and Method Details. Gene diagram not to scale.

(B) Gross phenotypes of $ZRS^{WT/\Delta}$ (top) and serpentized $ZRS^{cZRS/\Delta}$ (bottom) mice. Scale bars, 10 mm.

(C and D) Limb phenotypes of knockin mice with ZRS orthologs from other vertebrate species.

(C) Phylogeny and approximate divergence estimates (Amemiya et al., 2013; Hsiang et al., 2015; Wright et al., 2015) are shown on the left. Schematic mouse *Shh* loci with the ZRS replaced by orthologs from human (hZRS), python (pZRS), cobra (cZRS), and coelacanth fish (fZRS) are shown.

(D) Comparative *Shh* mRNA in situ hybridization analysis in knockin mouse embryos during forelimb bud development (first column). Per knockin line, the *Shh* transcript distribution was assessed in at least three independent mouse embryos. See Figure S4G for hindlimb bud analysis of *Shh* expression. Corresponding whole-mount E14.5 knockin mouse embryos (second column) and skeletal preparations at E18.5 (third and fourth columns) are shown; s, scapula; h, humerus; r, radius; u, ulna; fe, femur; fi, fibula; t, tibia; a, autopod. The genotypes of the embryos are $ZRS^{WT/\Delta}$ (mouse), $ZRS^{hZRS/\Delta}$ (human), $ZRS^{pZRS/\Delta}$ (python), $ZRS^{cZRS/\Delta}$ (cobra), and $ZRS^{fZRS/\Delta}$ (coelacanth fish). Arrow points to rudimentary digits in $ZRS^{pZRS/\Delta}$ embryos. Bottom embryo shows E14.5

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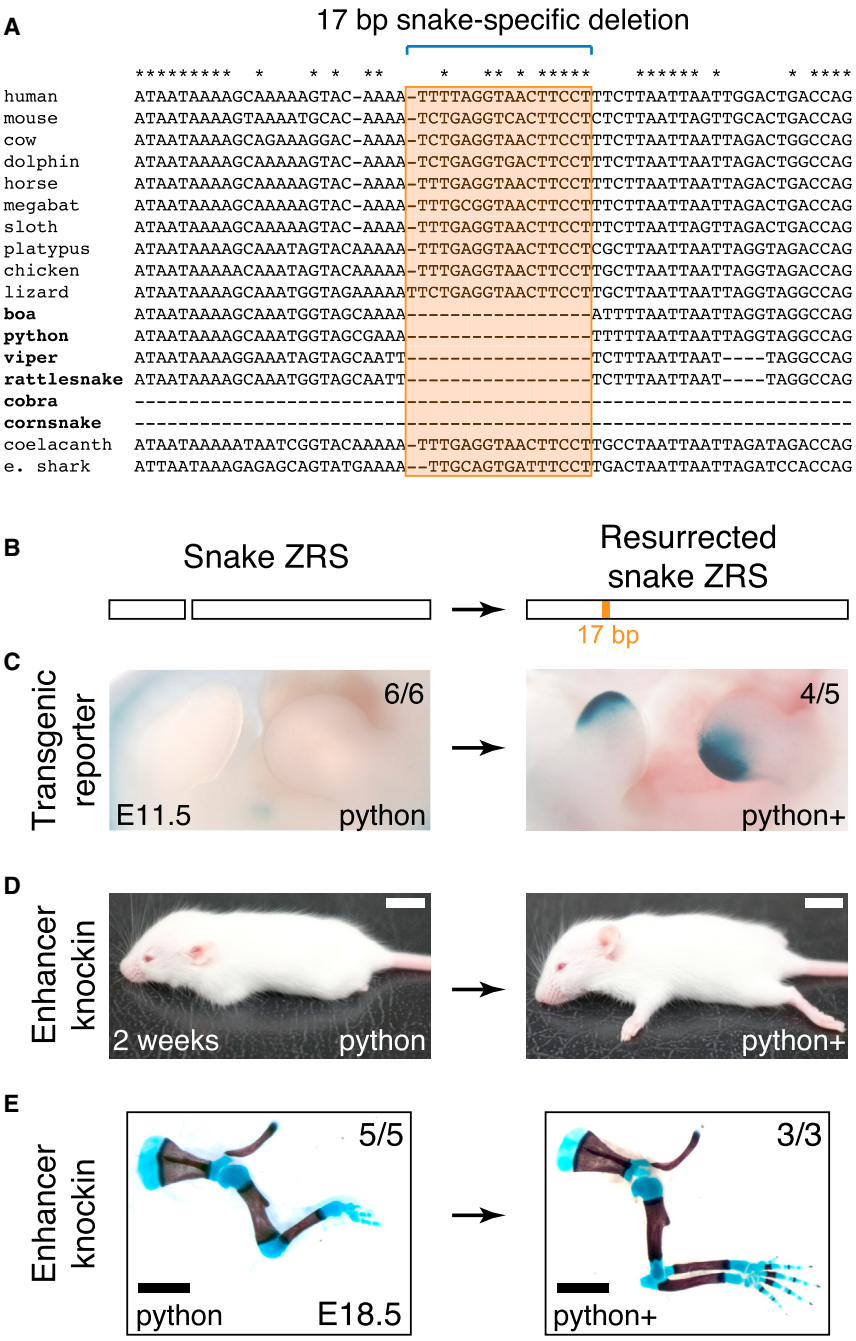

Figure 4. Resurrection of Snake Limb Enhancer Function In Vivo

(A) Snake-specific deletion in the ZRS. An alignment of the central ZRS region for 18 vertebrates, including six snakes, is shown. Asterisks indicate nucleotides that are conserved in limbed tetrapods and fish.

(B) A 17-bp sequence is able to resurrect python ZRS enhancer function.

(C) Shown are the wild-type (left) and modified (right) python ZRS in vivo enhancer activities in the limb buds of transgenic E11.5 mouse embryos. Numbers of embryos with lacZ activity in the limb over the total number of transgenic embryos screened are indicated.

(D) The resurrected allele is able to rescue limb development when knocked into the mouse genome in place of the wild-type ZRS. Shown are gross phenotypes of $ZRS^{pZRS/\Delta}$ (python, left) and $ZRS^{pZRS(r)/\Delta}$ (python+, right) mice at 2 weeks of age. Scale bars, 10 mm.

(E) Skeletal preparations from E18.5 knockin mice are shown. See Figures S5B and S5C for more detailed skeletal phenotypes. Scale bars, 2 mm.

See also Figure S5.

two to three rudimentary digits in the forelimb and a slightly enlarged ossification resembling a rudimentary zeugopod (Figure 3D). This result may be due to residual enhancer activity that was not detected in transgenic reporter assays (Figure 2). Consistent with this possibility, prolonged staining after RNA in situ hybridization indeed revealed very weak levels of *Shh* transcript in the posterior forelimb bud of python ZRS knockin mouse embryos (data not shown). Taken together our data indicate that both snake enhancers tested lost their ability to induce normal limb development in mice despite the much shorter evolutionary distance between mammals and snakes than between mammals and lobe-finned fish.

In Vivo Resurrection of a Distant-Acting Snake Limb Enhancer

To identify specific nucleotide changes within the enhancer that may have led to its loss of activity in snakes, we examined the snake sequences in detail. While multiple nucleotide differences are observed between snakes and limbed lizards (Figure S1), one small deletion of 17 bp stood out because it affected a region of the ZRS that was highly conserved across all examined tetrapods and fish (Figure 4A). Although it represents less than 10% of all sequence changes between the snake and lizard ZRS, this deletion is the only sequence that is deleted in all snakes but present in all examined limbed vertebrates and fish (Figures 4A and S1). To directly test whether this small snake-specific deletion contributed to the loss

gross and limb skeletal phenotypes of the $ZRS^{\Delta/\Delta}$ KO mice (see Figure S3 for details). Numbers of embryos that exhibited representative limb phenotype over the total number of embryos with the genotype are indicated. *Three of five mouse embryos displayed mild digit number variation (see Figures S4H–S4J). Scale bars, 0.1 mm (left column), 2 mm (columns 3 and 4).

See also Figures S3 and S4.

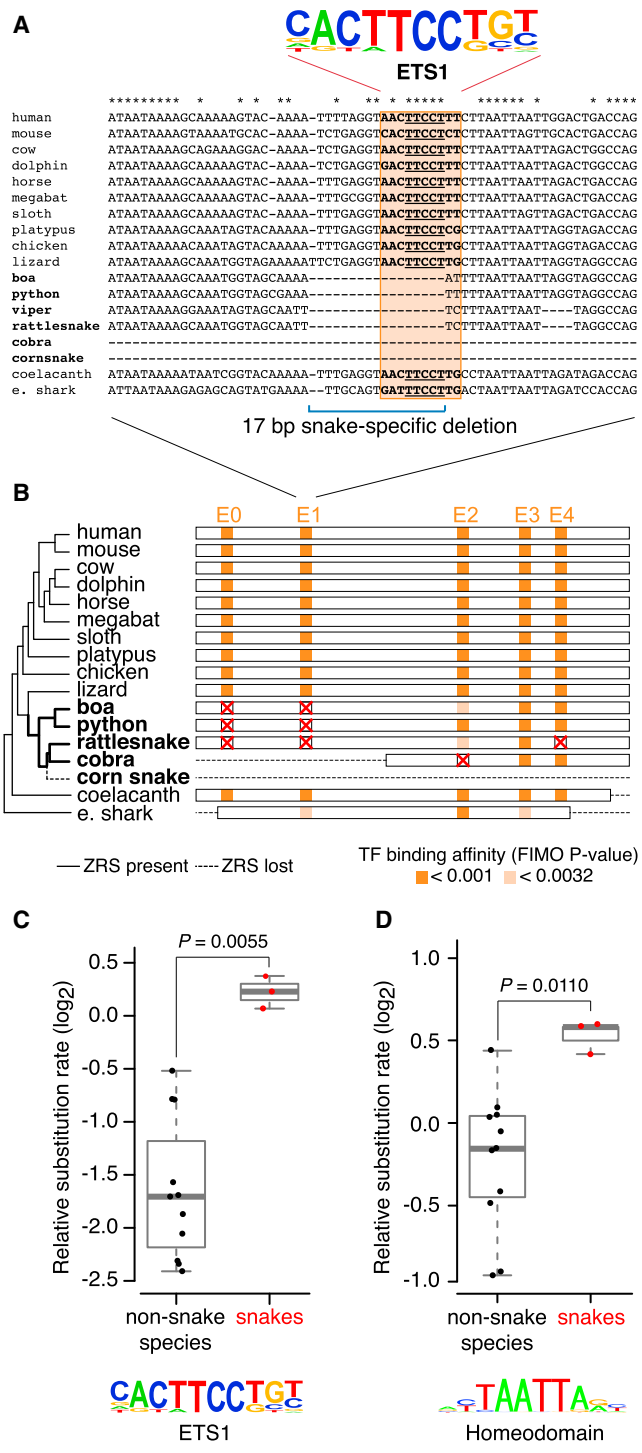

Figure 5. Loss of Conserved ETS Binding Sites in the Snake Lineage

(A) A detailed view of the E1 ETS binding site alignment for 18 vertebrates including six snakes. ETS1 motif is shown above. Asterisks indicate nucleotides that are conserved in limbed tetrapods and fish.

(B) Distribution of tetrapod conserved ETS motifs in the ZRS enhancer in different jawed vertebrates. Shown is a schematic alignment of the ZRS for 16 vertebrates (tree) and the locations of predicted ETS binding sites (E0–E4). Red crosses indicate motifs that were lost. See Figure S5 for details.

(C and D) Relative substitution rates in the ETS and homeodomain DNA motifs in the ZRS enhancer in non-snake species (black dots: species from Figure 5A) and snakes (red dots: boa, python and rattlesnake). Mann-Whitney p value is shown on top.

See also Figure S5.

of enhancer activity in snakes, we created a partially ancestral allele by reintroducing the 17-bp deleted sequence into the python enhancer sequence (Figures 2, 4A, and 4B). In a transgenic mouse reporter assay, this reintroduction of 17 bp of sequence alone was sufficient to reinstate full enhancer activity in the posterior mesenchyme of the limb bud at E11.5 (Figure 4C). To determine whether this allele could also functionally restore normal limb development in vivo, we used CRISPR/Cas9 genome editing to replace the endogenous mouse enhancer with this partially ancestral allele. Consistent with the results of the transgenic reporter experiments, the resulting knockin mice with the modified python allele had normal limbs (Figures 4D, 4E, and S5B). These results suggest that a 17-bp snake-specific deletion contributed to enhancer degeneration and that synthetic reintroduction of this microdeletion is sufficient to recreate the ancestral function of the ZRS and to rescue limb development in vivo.

To identify specific transcription factors that may be involved in the loss of enhancer function, we examined potential transcription factor binding sites that may have been affected by the 17-bp sequence deletion in the snake lineage. We identified a highly conserved motif within the deleted region whose sequence matched the binding preference of the ETS1 transcription factor. ETS1 has been suggested to directly activate the ZRS enhancer by binding to multiple ETS recognition sites (Lettice et al., 2012). We scanned the ZRS-orthologous sequences from 18 vertebrates for the presence of additional conserved ETS motifs (Figure S5C). In total, five ETS motifs within the enhancer are conserved across tetrapods, which includes four ETS binding sites previously identified in the mouse enhancer (Lettice et al., 2012). Remarkably, all five motifs were also conserved in coelacanth (bony fish), and three were present in elephant shark (cartilaginous fish, Figures 5 and S5C). In contrast to this strong conservation of ETS motifs across limbed vertebrates and fish, and, despite the overall conservation of the ZRS sequence in basal snakes, all examined snakes have lost the E0 and E1 ETS motifs. In addition, the E4 motif was lost in rattlesnake, and cobra lost the E2 motif (Figures 5B, S1, and S5C). More generally, in vertebrates with paired appendages the ETS sites show increased evolutionary constraint compared to the rest of the ZRS, whereas in snakes the ETS sites do not stand out as particularly constrained (Figure 5C). The fact that loss of the E1 motif in the mouse ZRS is not sufficient to alter limb bud expression (Lettice et al., 2012) and that the boa ZRS is active despite the absence of both E0 and E1 motifs indicate that loss of these motifs alone cannot explain ZRS deactivation in the snake lineage. We therefore also scanned the ZRS for other transcription factor motifs that showed a similar snake-specific loss of evolutionary constraint (Table S5). Interestingly, binding sites for homeodomain transcription factors, which have also been implicated in ZRS regulation (Capellini et al., 2006; Kmita et al., 2005; Lopez-Rios, 2016), display a similar

increase in substitution rate in snakes (Figure 5D). Taken together, our results implicate the loss of the E1 ETS site as well as potentially other ETS and homeodomain transcription factor binding sites in the loss of function of this limb enhancer in snakes.

DISCUSSION

In the present study, we demonstrate an increased rate of sequence changes, as well as progressive in vivo loss of function for a distant-acting limb enhancer in snakes. Decreased sequence conservation and loss of enhancer function were most pronounced in advanced snakes, which have lost all skeletal limb structures. The only snake genome in which no ZRS sequence was detected belonged to the corn snake. Our results indicate that the previously reported loss of the ZRS enhancer in Japanese rat snakes (Sagai et al., 2004), a member of the same subfamily (*Colubrinae*) as corn snakes, is not representative of snakes in general but affects only a small subset of advanced snakes where it occurred after the morphological loss of all limb structures (Figures 1B and S1). Across the snake species examined, the progressive sequence degeneration of the enhancer correlated with its loss of activity in transgenic reporter assays. In contrast, across all limbed tetrapods and fish examined, the enhancer activity was highly conserved. Remarkably, even a ZRS ortholog from fish (coelacanth), which shares less sequence similarity with the human ortholog than with the python ortholog (57% versus 59%), was sufficient for normal limb development despite the major morphological differences between mammalian limbs and coelacanth fins.

The molecular basis of loss of limbs in snakes as they evolved from their limbed ancestor has been the subject of extensive speculation (Apesteguía and Zaher, 2006; Cohn and Tickle, 1999; Di-Poï et al., 2010; Infante et al., 2015; Lopez-Rios, 2016; Martill et al., 2015; Sagai et al., 2004; Tchernov et al., 2000; Zeller et al., 2009). Our genomic enhancer replacement experiments in mice conclusively demonstrate that the loss of function in a single enhancer observed in snakes is sufficient to cause severe limb reduction in mice, raising the possibility that ZRS deactivation contributed to the loss of limbs in the snake lineage. However, changes in other sequences involved in limb development must also have occurred in snakes. These changes could for example involve regulation of *Hox* genes that act upstream of *Shh* (Cohn and Tickle, 1999; Di-Poï et al., 2010; Head and Polly, 2015), or other genes that are critical for initiation of limb development (e.g., Min et al., 1998; Rallis et al., 2003; Sekine et al., 1999; Tanaka et al., 2002). Notably, following the morphological disappearance of limbs, any sequence required exclusively for limb development is no longer subject to negative selection and is expected to degrade over time. This is exemplified by the reduction in the transgenic reporter activity of other serpentine limb enhancers whose phenotypic impact on limb development remains to be determined (Guerreiro et al., 2016; Infante et al., 2015). In the case of the ZRS, the enhancer activity observed in a basal snake (boa, Figure 2) suggests that the sequence degeneration of the ZRS in snakes started in conjunction with or, more likely, after other disruptive molecular events contributing to the loss of limbs. Consequently, we do not expect that the reintroduction of a fully functional ZRS into a snake genome alone would be sufficient to induce the formation of fully or even partially developed limbs in snakes.

While we deliberately focused on a locus with strong pre-existing evidence for function from human disease and mouse genetics studies (reviewed in Hill and Lettice, 2013; VanderMeer and Ahituv, 2011), an increasing number of unbiased genome-wide enhancer data across closely and distantly related animal species (Acemel et al., 2016; Arnold et al., 2014; Cotney et al., 2013; Eckalbar et al., 2016; Gehrke et al., 2015; He et al., 2011; Prescott et al., 2015; Reilly et al., 2015; Villar et al., 2015; Xiao et al., 2012) creates a rapidly growing list of candidate lineage- and species-specific enhancers. A major challenge is the identification of the subsets of these enhancers that functionally contribute to morphological and other phenotypic diversity. Our study provides an example how genome editing-enabled enhancer replacement makes it possible to recapitulate the functional erosion of a regulatory sequence across evolution through in vivo experiments. As genome-editing tools are becoming increasingly available, we expect that this approach will be useful to routinely study the phenotypes associated with evolutionary changes in other regulatory sequences associated with morphological adaptations in vertebrates.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.09.028.

An audio PaperClip is available at http://dx.doi.org/10.1016/j.cell.2016.09.028#mmc2.

AUTHOR CONTRIBUTIONS

E.Z.K., D.E.D., E.M.R., A.V., and L.A.P. conceived the project. E.Z.K. and O.K. performed the phylogenetic analysis. E.Z.K. and U.S.M. cloned transgenic reporter and targeting vectors. E.Z.K., B.J.M., I.P.-F., C.S.P., T.H.G., M.K., E.A.L., J.A.A., and V.A. carried out transgenic validation. E.Z.K. performed the enhancer knockout and knockin studies. V.T., J.L.-R., M.O., and E.Z.K. performed in situ hybridization (ISH). I.B. and E.Z.K. performed motif analysis.

E.Z.K., A.V., and L.A.P. wrote the manuscript with input from the remaining authors.

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Article

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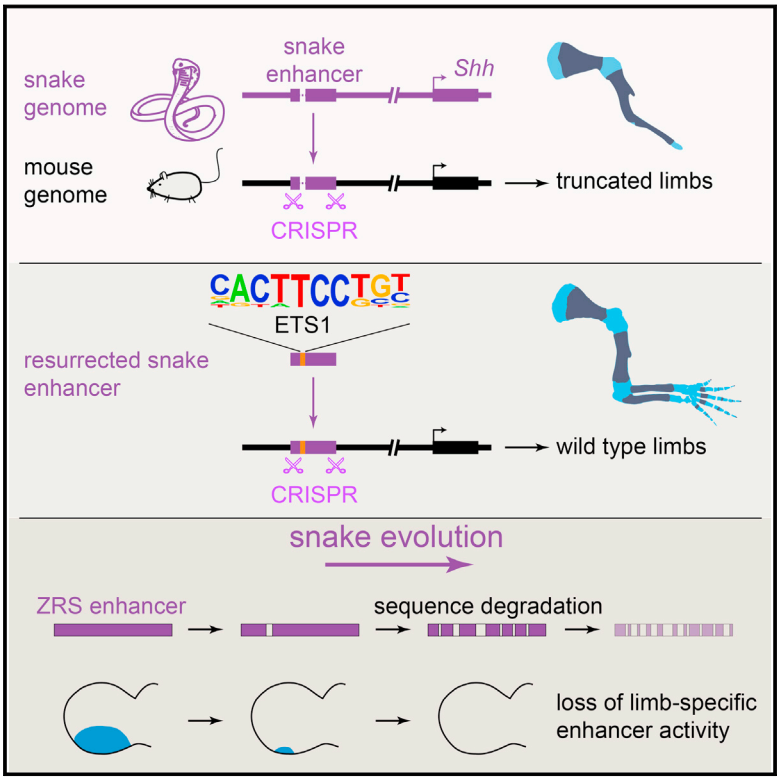
Progressive Loss of Function in a Limb Enhancer during Snake Evolution

Graphical Abstract



Highlights

- Activity of the critical ZRS limb enhancer is highly conserved across vertebrates
- ZRS enhancer has progressively lost its function during snake evolution
- Snake-specific nucleotide changes contributed to the loss of ZRS enhancer function
- Resurrection of snake enhancer function in vivo

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In Brief

Morphological disappearance of limbs in snakes is associated with sequence changes disrupting the function of a critical limb enhancer.

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