REVIEW

WILEY *genesis*
The Journal of Genetics and Development

Developmental, genetic, and genomic insights into the evolutionary loss of limbs in snakes

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Summary

The evolution of snakes involved dramatic modifications to the ancestral lizard body plan. Limb loss and elongation of the trunk are hallmarks of snakes, although convergent evolution of limb-reduced and trunk-elongated forms occurred multiple times in snake-like lizards. Advanced snakes are completely limbless, but intermediate and basal snakes have retained rudiments of hindlimbs and pelvic girdles. Moreover, the snake fossil record indicates that complete legs were re-acquired at least once, suggesting that the potential for limb development was retained in some limb-reduced taxa. Recent work has shown that python embryos initiate development of a transitory distal leg skeleton, including a footplate, and that the limb-specific enhancer of the *Sonic hedgehog* gene, known as the zone of polarizing activity regulatory sequence (ZRS), underwent gradual degeneration during snake evolution. In this article, we review historical and recent investigations into squamate limblessness, and we discuss how new genomic and functional genetic experiments have improved our understanding of the evolution of limblessness in snakes. Finally, we explore the idea that pleiotropy of *cis*-regulatory elements may illuminate the convergent genetic changes that occurred in snake-like lizards, and we discuss a number of challenges that remain to be addressed in future studies.

KEYWORDS
enhancer, Hox genes, limblessness, limb reduction, Sonic hedgehog, squamate

1 | INTRODUCTION

Evolutionary reduction and loss of limbs has occurred multiple times in every class of tetrapod (four-limbed) vertebrates. Limb reduction is often associated with other specializations that have allowed tetrapods to transition to new niches, such as the terrestrial-to-aquatic transition of cetaceans (Uhen, 2010), surface-to-burrowing habitats of fossorial lizards (Caldwell, 2003; Gans, 1975; Greer, 1991) and amphibians (Duellman & Trueb, 1986; Jenkins & Walsh, 1993; Wilkinson & Nussbaum, 2006), or the obligate bipedalism of flightless ratite birds (Yonezawa et al., 2017). Most examples of limb reduction are restricted to either the forelimbs or the hindlimbs; only a few tetrapod groups have undergone complete loss of both sets of limbs, and such cases are nearly always associated with body elongation. Some of the most extreme cases of elongated and completely limbless body plans are found in squamate reptiles, such as snakes (Caldwell, 2003; Gans, 1975) and snake-like lizards (Caldwell, 2003; Gans, 1975; Greer, 1991), and in the gymnophionan amphibians or caecilians (Duellman & Trueb,

1986; Jenkins & Walsh, 1993; Wilkinson & Nussbaum, 2006). For some of these cases, most notably snakes, the mechanisms of limb reduction are beginning to be understood at the developmental and the genomic levels (Kvon et al., 2016; Leal & Cohn, 2016). Moreover, new insights into the evolutionary history and phylogenetic relationships of snakes have allowed integration of paleontological, genomic, and developmental datasets, and these interdisciplinary analyses are beginning to uncover how the serpentiform body plan evolved. In this article, we review progress in these areas and discuss how such data sets can generate new testable hypotheses for future studies.

2 | THE ORIGIN OF SNAKE BODYPLAN

Snakes are one of the best-known and most successful examples of limbless vertebrates, forming an adaptive radiation of more than 3,600 species (Uetz, Freed, & Hošek., 2016) that contains extensive morphological, physiological, and ecological diversities (Vitt & Caldwell, 2014). The origin
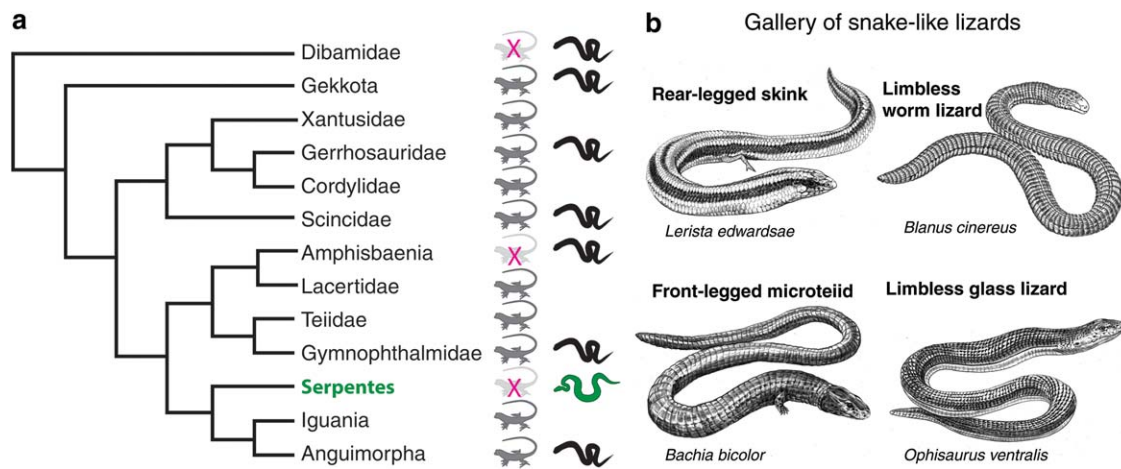
FIGURE 1 Convergent evolution of the serpentiform body plan in squamates. (a) Snakes (green) originated within a diverse lineage in which limb loss and body elongation evolved multiple times. Snake-like body plans evolved convergently in many lizard lineages (in black). The typical four-legged body plan (gray) co-existed in some groups but was lost in others (light gray with a red X). (b) Limb loss in snake-like lizards is diverse; forelimbs were lost in some groups (e.g., rear-legged skink), whereas hindlimbs were reduced in others (e.g., front-legged microteiid). Complete limblessness arose in some lineages, such as the surface dwelling glass lizard and the fossorial worm lizard. Phylogenetic relationships after Zheng and Wiens (2016) and body plans for each terminal are after Brandley et al. (2008) and Zug et al. (2001)

of the snake bodyplan has attracted the attention of biologists for centuries; however, only recently have advances in squamate paleobiology and phylogenomics begun to clarify their phylogenetic affinities and patterns of morphological evolution (Apesteguia & Zaher, 2006; Brandley, Huelsenbeck, & Wiens, 2008; Harrington & Reeder, 2017; Pyron, Burbrink, & Wiens, 2013; Tchernov, Rieppel, Zaher, Polcyn, & Jacobs, 2000; Zheng & Wiens, 2016). Morphological and molecular phylogenetic studies agree that snakes are closely related to lizards (Estes, De Queiroz, & Gauthier, 1988; Gauthier, Kearney, Maisano, Rieppel, & Behlke, 2012; Townsend, Larson, Louis, & Macey, 2004; Zheng & Wiens, 2016), and together they form the superlineage Squamata, indicating that the snake body plan evolved from a generalized four-legged lizard-like ancestor (Figure 1a). Within Squamata, the evolution of a serpentiform bodyplan is not exclusive to snakes (Figure 1); indeed, trunk elongation and limb reduction evolved independently in almost every major lineage of Squamata (Brandley et al., 2008; Greer, 1991; Wiens, Brandley, & Reeder, 2006). In most limb-reduced clades, forelimb reduction is more common than hindlimb reduction, although in many snake-like lineages, both the forelimbs and the hindlimbs are externally absent (Brandley et al., 2008).

The earliest relatively complete snake fossil belongs to the late Cretaceous snake *Najash rionegrina* (Apesteguia & Zaher, 2006; Zaher, Apesteguia, & Scanferla, 2009). No remnant of the forelimbs or the pectoral girdle is found in *N. rionegrina* (or in any living or fossil snake). By contrast, the hindlimb skeleton is relatively complete, conserving the stylopod (femur) and zeugopod (tibia and fibula) and pelvic girdle. *Najash* demonstrates that forelimbs were lost first, yet hindlimbs persisted for millions of years, existing as rudimentary structures in extant basal and intermediate snakes (Apesteguia & Zaher, 2006; Brandley et al., 2008; Greer, 1991; Houssaye et al., 2011; Tchernov et al.,, 2000; Wiens, Brandley, & Reeder, 2006; Zaher et al., 2009). The pelvic girdle of *Najash* is similar to that of limbed lizards, sharing features such as an articulation with the axial skeleton by means of two sacral vertebrae, and a location outside of the ribcage (Apesteguia & Zaher, 2006; Zaher

et al., 2009). In extant snakes with hindlimb rudiments, the pelvic girdle is located inside the ribcage and has no articulation with the axial skeleton (sacral vertebrae are absent). Moreover, hindlimbs consist only of a distally truncated femur capped by a terminal claw (Apesteguia & Zaher, 2006; Zaher et al., 2009). After the divergence of the basal and intermediate snake lineages, advanced snakes (caenophidians) lost all remnants of the pelvic and hindlimb skeleton (Bellairs & Underwood, 1951; Brandley et al., 2008; Harrington & Reeder, 2017).

3 | AXIAL AND APPENDICULAR EVOLUTION IN SNAKES

The transformation of a four-legged lizard ancestor into a serpentiform organism involved the evolution of body elongation and limb loss (Bellairs & Underwood, 1951; Gans, 1975). The positive correlation of body length with limb reduction has been explored within Squamata (Brandley et al., 2008; Wiens & Slingluff, 2001); however, whether this correlation is caused by a release of the selective pressure to maintain fully formed limbs, due to a major change in mode of locomotion, or by a developmental tradeoff between axial and appendicular developmental programs, is not yet clear. Body elongation has also been connected to an apparent reduction in regionalization of the snake axial skeleton (Cohn & Tickle, 1999). Anteroposterior patterning of the vertebral column is regulated by developmental regionalization of the vertebrate embryo by nested expression of *Hox* genes in the paraxial mesoderm (Burke, Nelson, Morgan, & Tabin., 1995; McIntyre et al., 2007; Vinagre et al., 2010; Wellik & Capecchi, 2003), whereas developmental positioning of limbs depends on this axial regionalization of *Hox* gene expression in the lateral plate mesoderm (Cohn et al., 1997; Minguillon et al., 2012; Nishimoto, Minguillon, Wood, & Logan, 2014; Nishimoto, Wilde, Wood, & Logan, 2015). The nature of the interactions between paraxial and lateral plate mesoderm during limb induction is not entirely understood (Kieny, 1969; Nishimoto et al., 2015; Pinot, 1970), and it is

possible that changes in the regulation of *Hox* gene expression along the primary body axis could affect axial skeletal regionalization as well as limb development.

Despite the correlation found between body elongation and limb reduction/loss, the possibility of a mechanistic relationship between these developmental processes remains to be determined. Elongation of the axial skeleton can be achieved by two general mechanisms, increasing vertebral size and/or increasing vertebral number (Gomez & Pourquie, 2009; Parra-Olea & Wake, 2001). Snake body elongation was achieved by a dramatic increase in vertebral number caused by higher oscillation rate of the segmentation clock in the unsegmented paraxial mesoderm (Gomez et al., 2008). Similarly, the pluripotency factor *Oct4* is maintained for a longer developmental time in snake embryos, probably due to a rearrangement of the transcriptional regulatory landscape upstream of *Oct4*. Indeed, when mice were engineered to overexpress *Oct4* for a longer developmental time (under the control of a *Cdx2* enhancer), the *Cdx2-Oct4* mice developed longer axial skeletons (Aires et al., 2016). The marked elongation of the trunk region reflected a delayed activation of posterior *Hox* genes (Aires et al., 2016). Similarly, the timing of *Gdf11* expression in the unsegmented paraxial mesoderm determines hindlimb/sacral positioning; modulation of *Gdf11* results in extension of the precloacal axial skeleton (McPherron et al., 1999). Gdf11 activity coordinates limb induction with axial integration—specification of the sacral region—by regulating *Hox* genes in paraxial and lateral plate mesoderm (McPherron et al., 1999; Matsubara et al., 2017). Downregulation of *Gdf11* by RNA interference in the paraxial mesoderm prevents the activation of *Tbx4* expression during chicken hindlimb initiation, and pharmacological inhibition of the GDF11 receptor (ALK5) results in downregulation of *Tbx4*, *Fgf10*, and *Islet1* in lateral plate mesoderm (Matsubara et al., 2017). Reciprocally, precocious activation of GDF11 in the lateral plate mesoderm in chicken embryos results in rostral displacement of hindlimb position (Matsubara et al., 2017). Consistent with the effects of GDF11 manipulations in mouse and chick embryos, analysis of *Gdf11* expression in snakes revealed a striking delay in the activation of *Gdf11* transcription in the paraxial mesoderm (relative to limbed vertebrates), suggesting that heterochronic changes in *Gdf11* activation in the paraxial mesoderm determines hindlimb/sacral positioning and trunk length (McPherron et al., 1999; Matsubara et al., 2017).

The aforementioned studies address axial elongation; however limb loss is snakes is also associated with changes in axial skeletal regionalization. In the first analysis of HOX protein expression in python embryos, it was reported that the expression domains of middle-group *Hox* genes, which mark the position of forelimbs and the neck/thorax boundary in limbed tetrapods, were shifted anteriorly (Cohn & Tickle, 1999). This suggested an anterior expansion of thoracic and flank (the limbless region between forelimbs and hindlimbs) identity in snakes, which could explain the absence of forelimbs (Cohn & Tickle, 1999). A subsequent analysis of *Hox* gene expression in corn snakes (a more derived snake relative to pythons) showed that their middle-group *Hox* genes were similar to the expression patterns seen in four-legged lizards (Woltering et al., 2009). Whether the differences in *Hox* expression domains observed in these two snakes reflect technical differences in the experimental approaches (antibodies vs. mRNA probes) or lineage-specific differences in *Hox* gene regulation will require further studies. It is noteworthy, however, that morphometric analysis revealed fine-grained morphological regionalization of snake vertebral columns, which challenges the assumption of a non-regionalized snake axial skeleton and is consistent with positional differences being encoded by the embryonic snake Hox code (Head & Polly, 2015). By contrast to the idea that snake axial patterning reflects upstream (*cis-*) changes in *Hox* gene regulation, there is some evidence that mutations in the coding sequence of posterior *Hox* genes, as well as the enhancers they bind, played a role in the evolution of the snake trunk (Di-Poi et al., 2010; Guerreiro et al., 2013). Functional studies in mice showed that the snake regulatory sequences prevent Hox10 rib-repression and lead to development of an elongated ribcage in place of lumbar vertebrae (Guerreiro et al., 2013). Further studies will be needed to determine whether snakes underwent similar modifications to limb-specific enhancers (and/or the coding sequences) of *Hox* genes and, if so, whether such mutations could disrupt limb bud development.

4 | FORELIMB LOSS IN SNAKES AND SNAKE-LIKE LIZARDS

No known fossil or extant snakes retain remnants of a pectoral skeleton, nor are there any known examples of forelimb bud initiation in embryos (Raynaud, 1985). Therefore, little is known about the evolutionary history or molecular basis of forelimb reduction in snakes. Although it is not yet possible to pinpoint when forelimbs were lost during snake evolution, the complete absence of pectoral girdle and forelimb remnants in basal and derived snakes probably reflects a total absence of forelimb bud induction or initiation (see below).

In limbed tetrapods, normal forelimb and hindlimb initiation depends on the activation of *Fgf10* in the limb-forming regions of the lateral plate mesoderm (Min et al., 1998; Sekine et al., 1999). Fgf10 signaling results in activation of *Fgf8* in the overlying ectoderm and initiation of limb outgrowth. Shortly after limb buds emerge, the distal limb bud epithelium forms the apical ectodermal ridge (AER), which secretes multiple Fgf ligands to sustain proximodistal outgrowth of the limb bud (Min et al., 1998; Niswander, Jeffrey, Martin, & Tickle, 1994; Ohuchi et al., 1997). T-box family transcription factors *Tbx5* and *Tbx4* are critical for activation of *Fgf10* in the lateral plate mesoderm and for induction of forelimbs and hindlimbs, respectively (Agarwal et al., 2003; Naiche & Papaioannou, 2003; Rallis et al., 2003).

Recent studies in chickens demonstrate that the activation of *Tbx5* at forelimb levels depends on communication between somitic and lateral plate mesoderm (Nishimoto et al., 2015). Retinoic acid (RA) and Wnt/β-catenin signaling, together with Hox (four and five paralogs) transcription factors in the lateral plate mesoderm activate *Tbx5* transcription (Minguillon et al., 2012). Therefore, correct specification and positioning of forelimbs is achieved by transactivation of a *Tbx5* forelimb-specific enhancer by Hox, RARα, and β-catenin transcription factors (Nishimoto et al., 2015). Binding of caudal HOX proteins to the *Tbx5* forelimb enhancer, on the other hand, represses *Tbx5*
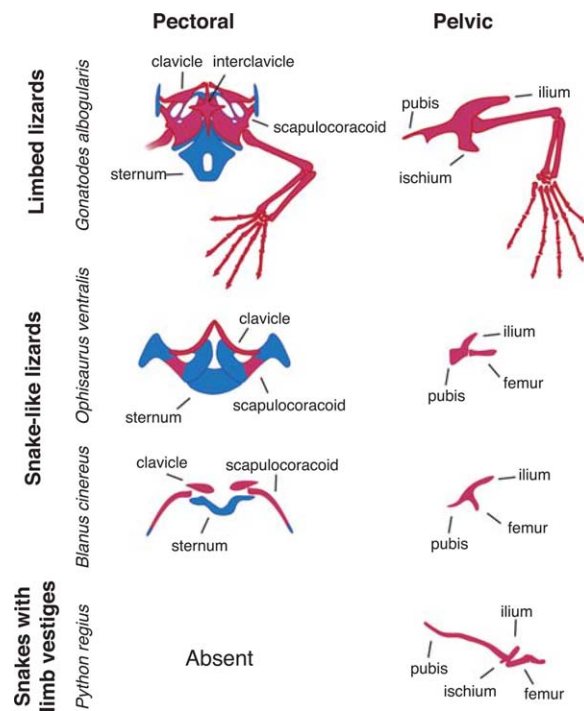
FIGURE 2 Examples of normal and reduced pectoral and pelvic skeletons in squamates. Comparison of the pectoral and pelvic skeletons (bone in magenta, cartilage in blue) in a four-legged lizard, two snake-like lizards, and a snake with limb vestiges (note the rudimentary femur in the last three). *Ophisaurus ventralis* skeleton from Cope (1892), *Blanus cinereus* from Kearney (2002), *Python regius* from Leal & Cohn, (2016) and *Gonatodes albogularis* from F. Leal (unpublished)

transcription, delimiting the posterior *Tbx5* boundary in the flank (Nishimoto et al., 2014).

Although forelimb loss is poorly understood in snakes, reduction of the forelimbs has occurred multiple times in squamate evolution, and most snake-like lizards conserve at least part of the pectoral girdle (Figure 2; Camp, 1923; Cope, 1892; Kearney, 2002; Stephenson, 1962; Stokely, 1947). This apparent dissociation of the forelimb and the pectoral girdle skeletons is especially interesting in light of the phenotypes of *Tbx5* and *Fgf10* conditional null mice. *Tbx5* conditional mutants demonstrate the critical function of *Tbx5* not only for forelimb induction (mutants lack forelimb buds), but also for formation of the pectoral girdle, which fails to develop (Bickley & Logan, 2014; Rallis et al., 2003). By contrast, *Fgf10* conditional mutants, which are limbless, nonetheless initiate formation of small limb buds, but these fail to develop beyond the bud stage. Moreover, the phenotype of the pectoral girdle skeleton in *Fgf10* mutants is less severe than in *Tbx5* mutants, as the former develops a scapula, clavicle, and sternum (Bickley & Logan, 2014; Min et al., 1998; Sekine et al., 1999). Experimental studies of limb development allow formulation of hypotheses that can be tested directly through comparative developmental and genomic analyses. For example, in glass lizards (*Ophisaurus ventralis*) and slow worms (*Anguis fragilis)*, the humerus and all distal elements are completely absent (Figure 2) but they have a complete pectoral girdle and develop very small but transitory forelimb buds (Cope, 1892; Raynaud, 1985; Raynaud, 1990).

Based on the studies described above, it is tempting to speculate that snake-like lizards with pectoral girdle elements could show conservation of *Tbx5* activity but diminished *Fgf10*, whereas those lacking pectoral elements altogether might have lost the pectoral domain of *Tbx5*. Nonetheless, it is important to note that genetic studies in model systems are not necessarily reliable predictors of the mechanisms underlying evolutionary reduction of limbs in non-model organisms, and the comparative studies performed to date demonstrate that vertebrates have taken numerous paths to fin/limb loss (Leal & Cohn, 2016; Lin et al., 2016; Shapiro, Bell, & Kingsley, 2006; Tanaka et al., 2005). Therefore, each case of limb reduction merits direct investigation into the underlying mechanisms, rather than inference from model organisms or parallel cases of convergence.

In snakes, the failure of forelimb budding and the absence of pectoral girdles might suggest that the *Tbx5-Fgf10* pathway is not activated in snake embryos, but analysis of corn snake embryos showed that *Tbx5* is actively transcribed in a broad anteroposterior domain of lateral plate mesoderm (Woltering et al., 2009). Corn snake embryos have broad *Tbx5* expression in the somatic layer of the lateral plate mesoderm (LPM), extending from the level of the heart down to the cloaca/genitalia (Woltering et al., 2009). In limbed tetrapods, the forelimb domain of *Tbx5* in the lateral plate mesoderm is controlled by HOX proteins (Minguillon et al., 2012). In snakes, the expression domain of *Tbx5* suggests that the interaction of HOX proteins and *Tbx5* regulatory elements has been disrupted. Although the developmental genetic causes of expanded expression of *Tbx5* in snake embryos still needs to be understood, its persistence in lateral plate mesoderm (splanchnic and somatic) likely reflects a degree of constraint imposed by the essential non-limb related functions of *Tbx5*, such as in cardiac development (Koshiba-Takeuchi et al., 2009).

The scarcity of forelimb or pectoral girdle skeletons in the snake fossil record does not necessarily indicate that forelimb reduction happened abruptly. Given that snake-like lizard lineages underwent gradual vestigialization of forelimbs before they were lost altogether, there are unlikely to be developmental constraints precluding intermediate forms of forelimb reduction (Brandley et al., 2008). Disruption of RA activity in the lateral plate mesoderm in *Rdh10*^trex mouse mutants leads to reduced activation of *Tbx5* transcription in the lateral plate mesoderm, which results in formation of size-reduced limb buds, and, ultimately, a forelimb-reduced phenotype. Similarly, delayed activation of *Tbx5* transcription in the lateral plate mesoderm can lead to size-reduced forelimbs and pectoral girdles (Bickley & Logan, 2014). Therefore, reduced and/or delayed transcriptional activation of *Tbx5* may affect recruitment of forelimb progenitor cells that will form the limb, thereby causing reduction in limb bud and forelimb skeleton size, and some researchers have suggested that this could account for wing reduction in flightless birds (Bickley & Logan, 2014). A Tbx5-mediated deficiency in the number of limb progenitor cells could be generated at early limb bud outgrowth stages or as early as limb bud induction, as the Tbx5-Fgf10 pathway has been implicated in the localized epithelial-to-mesenchymal-transition of the epithelial somatopleure that contributes to the production of forelimb mesenchymal progenitor cells (Gros & Tabin, 2014). The diversity of forelimb-reduced vertebrates from which

eggs can be collected means that there are many rich opportunities for future investigations into the mechanism of forelimb reduction during vertebrate evolution.

5 | HINDLIMB LOSS IN SNAKES AND SNAKE-LIKE LIZARDS

Presence of hindlimb vestiges in extinct and extant basal snakes indicates that hindlimbs underwent reduction before being eliminated (Apesteguia & Zaher, 2006). Moreover, the fossil record of snakes suggests that the onset of hindlimb reduction followed loss of forelimbs. Although there are examples of pelvic appendage reduction in squamates that retain complete forelimbs (the amphisbaenian *Bipes* and gymnophthalmid *Bachia*), forelimb reduction is more common that reduction of hindlimbs (Brandley et al., 2008). Why are hindlimbs more likely than forelimbs to be retained in squamates? Is there a developmental constraint that could explain this disparity between the frequency of forelimb and hindlimb loss during evolution?

Although in some cases, selective pressures likely stabilized hindlimb vestiges after they became coopted for copulatory behaviors (Gillingham & Chambers, 1982; Slip & Shine, 1988), the comparatively low frequency of hindlimb relative to forelimb reduction in many snake-like lizards and basal snakes might indicate the presence of a developmental constraint. One such constraint could be the developmental linkage between the hindlimb and the cloacal/genital fields. Cell lineage analyses of hindlimb buds and cloacal/external genital organs in chickens, mice, and lizards revealed these appendages arise from adjacent or even partially overlapping populations of cells (Herrera & Cohn, 2014; Tschopp et al., 2014; Valasek, Evans, Maina, Grim, & Patel, 2005). Indeed, the lateral plate mesoderm at the pelvic/cloacal level appears to be regionalized along the mediolateral (future dorsoventral) axis into dorsal hindlimb, ventral hindlimb, and external genital compartments. As the body wall closes, the lateral-most cells of the external genital fields are brought together at the ventral midline, where they form the paired genital swellings (Herrera & Cohn, 2014). The paired genital swellings then fuse to form a single medial phallus or remain unfused to form the paired hemipenes in squamates (Gredler, Sanger, & Cohn, 2015; Leal & Cohn, 2015). It remains to be determined whether failure of hindlimb budding in advanced snakes reflects loss of hindlimb field specification, or, alternatively, loss of the signal that initiates budding of cells in a hindlimb field. If the former scenario applies, then this would suggest that specification of hindlimb and external genital fields can be decoupled, as advanced snakes lack hindlimb buds but retain hemipenes, whereas the latter scenario would indicate retention of a prospective hindlimb field in advanced snakes.

Studies of muscle development identified a further link between the pelvic appendages and external genitalia. Analyses of cloacal and hindlimb muscle development in chickens revealed that cloacal muscles are derived from a population of myogenic progenitor cells that first enter the hindlimb bud and then migrate out of the limbs to populate the cloacal region (Valasek et al., 2005). Similarly, pectoral muscle progenitors initially migrate from the myotome into the forelimb bud and then migrate back out to form the pectoral muscles (Valasek et al.,

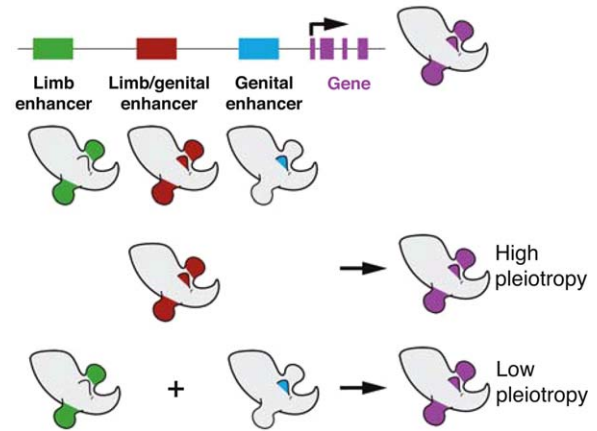**Enhancer modularity for limb-genital shared gene expression**

FIGURE 3 Enhancer modularity and pleiotropy in limb and genital gene expression. Limb and genital development share the expression of numerous genes. This shared gene expression can be achieved by the action of either lower modularity limb/genital enhancers or by the modular action of limb-specific and genital-specific enhancers. Theoretically, low modularity enhancers bear a higher pleiotropy load, whereas high modularity tissue-specific enhancers should have lower pleiotropy and higher tolerance for evolutionary modifications

2011). This developmental dependency of pectoral and cloacal muscles in the forelimb and hindlimb regions has been coined the "in–out mechanism" (Evans, Valasek, Schmidt, & Patel, 2006; Valasek et al., 2011). Just as loss of the forelimb buds would be expected to affect development of the pectoral musculature, loss of hindlimb buds should affect the myogenic lineage that forms the pelvic and cloacal musculature. Therefore, it will be interesting to determine how the cloacal and external genital musculature develops in the absence of hindlimb buds in advanced snakes.

Recent genome-wide comparisons of gene expression and enhancer activity during limb and genital development have identified further similarities at the transcriptomic (Tschopp et al., 2014) and epigenetic (Infante et al., 2015) levels. ChIP-seq analysis of active enhancers (using H3K27ac histone modification) in lizard limbs and genitalia demonstrated conservation of many enhancer sequences between these two organ primordia (Infante et al., 2015). The discovery that limbs and genitalia share numerous enhancers highlighted a new level of developmental pleiotropy, suggesting that there may be limits to the modular control of limb and external genital *cis*-regulatory elements (Figure 3). Interestingly, this developmental connection may have shaped the way snake genomes evolved, since many "limb enhancer" sequences have been conserved in snakes despite limb vestigialization (Infante et al., 2015). This conservation likely reflects the dual functions of these *cis*-regulatory elements in external genital development (Leal & Cohn, 2015).

Taken together, discoveries of mechanistic and regulatory links between hindlimb and external genital/cloacal development demonstrate that evolutionary reduction of hindlimbs may have been constrained by pleiotropic effects on cloacal and genital development. In
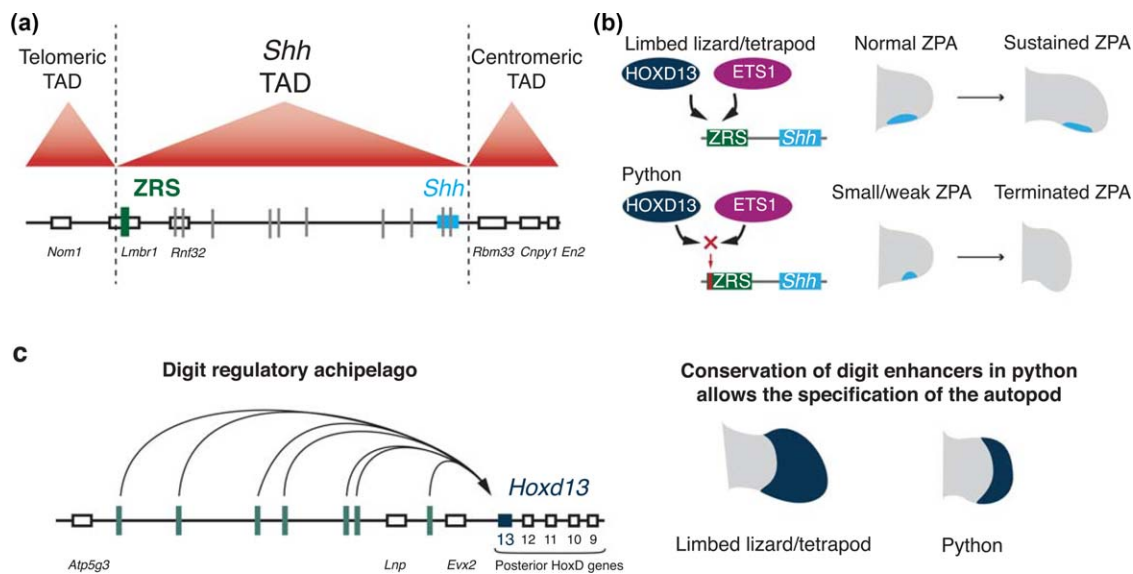
FIGURE 4 Evolution of limb loss by degeneration of the ZPA regulatory sequence (ZRS) and conservation of a molecular autopod in snakes. (a) Despite the ZRS being located about 1 Mb from the *Shh*, transcriptional activation is possible since the ZRS (green bar) and *Shh* (light blue rectangle) are located in the same TAD (*Shh* TAD), which also contains other organ-specific *Shh* enhancers (gray bars). This structure allows physical interaction of the ZRS and the *Shh* promoter to the exclusion of other promoters outside the *Shh* TAD. (b) Hindlimb loss in snakes is caused by three deletions in the ZRS, which reduce *trans*-activation of the enhancer by Hoxd13 and Ets1 transcription factors, leading to a small and weak *Shh* transcription (light blue) in the python ZPA (bottom) compared to a four-legged lizard (top). (c) Despite the premature termination of the python ZPA activity, the weak and brief exposure of the python limb bud to SHH is sufficient to specify a digit forming domain (autopod) by *Hoxd13* expression (dark blue) in the python limb bud. This autopodial domain of *Hoxd13* expression is the result of conservation of the digit-genital enhancers located in a gene desert centromeric to the *Hoxd* genes cluster. The *Shh* regulatory landscape was adapted from Anderson et al. (2014) and the Hoxd regulatory landscape, python ZRS, and limb development schematic were adapted from Leal and Cohn (2016)

advanced snakes, which are completely limbless, these pleiotropies were eventually circumvented in order to completely emancipate the external genitalia from hindlimb development, allowing hemipenes to be retained despite absence of hindlimb buds or any remnants of the legs. How these systems were decoupled is an open question.

6 | SNAKE HINDLIMB LOSS BY MUTATIONS IN THE ZRS, A LIMB-SPECIFIC ENHANCER OF *SHH*

The oldest known snake fossil with an intact postcranium, *N. rionegrina*, indicates that elongation of the trunk and loss of forelimbs preceded reduction of the hindlimbs (Apesteguia & Zaher, 2006). Extant blindsnakes, pythons, and boas retain a pelvic girdle and distally truncated femora (Figure 2). A recent analysis of python embryonic development showed that the hindlimb skeleton progress beyond femur development; cartilage condensations of the tibia, fibula and footplate form *in ovo*, but these structures are transitory, degenerating before hatching (Leal & Cohn, 2016).

Previous studies suggested that the python hindlimb bud was effectively "dead on arrival", because neither a morphological AER nor SHH protein, indicative of a zone of polarizing activity (ZPA), could be detected (Cohn & Tickle, 1999). Sagai et al. (2004) reported that snakes lack the limb-specific enhancer of *Shh*, known as the ZPA regulatory sequence (ZRS; Figure 4a), leading them to hypothesize that the ZRS

was lost in snakes and that this may have led to the evolution of limblessness. However, when python posterior hindlimb bud mesenchyme was transplanted under the AER of chick limb buds, SHH was expressed in the python tissue, which, in turn, induced duplication of chick digits (Cohn & Tickle, 1999). The finding that python hindlimb bud cells are competent to express *Shh* when exposed to permissive cues suggested that a ZRS sequence was likely to exist in the python genome.

More recent work examined python embryos at earlier stages of hindlimb development and showed that a transient burst of *Shh* expression could be detected in the hindlimb buds within the first 24 hr of oviposition (Leal & Cohn, 2016). *Shh* transcription in python hindlimb buds is weak and highly transient compared to *Shh* expression in limbed vertebrates (Figure 4b), although the pulse of *Shh* activity is sufficient to activate hedgehog signaling pathway, as indicated by expression of the hedgehog target genes *Ptch1* and *Gli1* in the posterior margin of the python hindlimb bud (Leal & Cohn, 2016). An AER forms in python hindlimb buds, but it too is transitory, degenerating from posterior-to-anterior shortly after cessation of *Shh* expression (Leal & Cohn, 2016). Studies of chicken limb development showed that sustained *Shh* expression is critical for the maintenance of the AER. Activation of the *Shh* pathway in limb bud mesenchyme induces expression of the bone morphogenetic protein (BMP) inhibitor Gremlin, which counteracts the AER-repressive activity of BMPs from the limb mesenchyme (Zuniga, Haramis, McMahon, & Zeller, 1999). Indeed, in python

hindlimb buds, *Gremlin* expression fades away after *Shh* expression is prematurely terminated (Leal & Cohn, 2016). These results indicate that the early arrest of *Shh* transcription in the python hindlimb bud breaks the ZPA–AER feedback loop, causing a premature regression of the AER and, ultimately, a precocious arrest of limb development.

A combination of comparative genomics in snakes and functional genetic tests in mice has begun to reveal the relationship between ZRS sequence evolution and limb reduction in snakes. In pythons, the nucleotide sequence of the ZRS is generally conserved, and its function has been retained to a limited degree (Kvon et al., 2016; Leal & Cohn, 2016). Analysis of advanced snakes, however, showed that the ZRS is barely recognizable, indicating that the enhancer became further degenerated in caenophidian snakes after they diverged from pythons (Kvon et al., 2016; Leal & Cohn, 2016).

Evolutionarily, the ZRS is a highly conserved sequence in tetrapods, where even single nucleotide mutations can cause misexpression or complete ablation of *Shh* expression in the limb (Lettice et al., 2003; Lettice, Hill, Devenney, & Hill, 2008). It has been proposed that the function of the ZRS shows a bipartite organization, in which the 5′ end codifies the spatial and temporal information and can be *trans*-activated at the ZPA, and the 3′ end is important for looping of the chromatin for the activation of the *Shh* promoter (Lettice et al., 2014). In basal snakes, three deletions in the 5′ end of the ZRS have compromised the transactivation potential of the ZRS. Given the conservation of the remainder of the gene regulatory network in python hindlimb development, it seems likely that diminished *Shh* expression caused by deletions in the ZRS played a central role in the evolutionary reduction of snake hindlimbs (Leal & Cohn, 2016). Therefore, the picture that is emerging from these studies is that early truncation of snake hindlimb development was caused by deletions in the 5′ ZRS that rendered the enhancer hypofunctional in the ZPA (Leal & Cohn, 2016).

Investigations of snake ZRS functions using transgenic mice have begun to connect sequence evolution to developmental activity *in vivo*. Tests of python and limbed lizard ZRS sequences using LacZ reporter constructs in transgenic mice embryos showed that the python ZRS is only weakly activated and restricted to a small domain of cells in the mouse ZPA (Leal & Cohn, 2016). When the python mutations were introduced into the mouse ZRS by site-directed mutagenesis, activity of the ZRS was significantly reduced (Leal & Cohn, 2016). Activation of *Shh* transcription in cells at the posterior margin of the mouse limb bud depends on the positive regulatory input of Hoxa/d, ETS, and Hand2 transcription factors, some which bind directly to the ZRS (Capellini et al., 2006; Galli et al., 2010; Lettice et al., 2012; Osterwalder et al., 2014). Protein–DNA binding and transactivation assays showed that the mutations present in the python ZRS disrupt sites needed for Ets1 and Hoxd13 binding (Figure 4b) and, in turn, for transactivation of the ZRS (Leal & Cohn, 2016).

In an elegant test of the functional consequences of the python ZRS deletions *in vivo*, Kvon et al. (2016) used CRISPR-Cas9 to replace the mouse ZRS sequence with the python sequence containing the deletions. The resulting mice developed dramatic distal limb truncations that resembled the python adult limb morphology, demonstrating that the deletions in the python ZRS are sufficient to disrupt transcription of *Shh* and development of limbs (Kvon et al., 2016). When they restored a 17-bp deletion in an Ets1 and Hoxd13 binding site, Kvon et al. (2016) were able to rescue normal limb development in the engineered mice, thereby demonstrating how microdeletions in critical regions of the ZRS can have dramatic effects on limb development.

Whether reduction of hindlimbs during the evolution of snakes was caused solely by ZRS mutations will require further studies, including additional comparisons of snake genomes. The studies performed to date do not exclude the possibility that hindlimb loss in snakes occurred by a different mechanism, and that the mutations in the ZRS reflect degradation that resulted from a release of selective pressures to maintain its function. However, degenerative mutations in the python ZRS do negatively impact *Shh* transcription, whereas other components of the limb developmental program, both upstream and downstream of *Shh* signaling, are activated during early stages of python hindlimb development, and the downstream factors switch off only after *Shh* transcription has been terminated. Therefore, the evidence obtained to date suggests that the premature arrest of *Shh* transcription in python hindlimb ZPA cells is, in principle, sufficient to explain the arrest of python hindlimb development.

7 | CHROMATIN ORGANIZATION AT THE *SHH* AND ZRS LOCI

Evolutionary modulation of *Shh* activity during limb development could be related to evolution of chromatin topology at the *Shh* and ZRS loci. Despite the large distance between the *Shh* promoter and the ZRS (∼1 Mb; Lettice et al., 2003), physical contact between those two elements is achieved by folding of the chromatin along this expanse which allows the physical interaction that initiates *Shh* transcription in the ZPA (Amano et al., 2009; Williamson, Lettice, Hill, & Bickmore, 2016). The ZRS and *Shh* are located at opposite ends of the topological association domain (TAD) that contains the other organ/tissue specific *Shh* enhancers (Figure 4a; Anderson, Devenney, Hill, & Lettice, 2014; Symmons et al., 2016). The ZRS is located at the telomeric end and *Shh* is at the centromeric end of the TAD, making the ZRS the furthest enhancer from the *Shh* promoter (Anderson et al., 2014; Symmons et al., 2016). Interestingly, the absolute distance of the ZRS to the *Shh* promoter does not seem to affect *Shh* transcriptional activation; however, when the ZRS is translocated to a position outside the boundaries of the TAD, then the ZRS is unable to interact with the *Shh* promoter and *Shh* fails to be transcribed at the ZPA. These results indicate that chromatin topology is a crucial parameter in the regulation of *Shh* transcriptional activation (Symmons et al., 2016).

Human limb malformations have been linked to mutations affecting TAD boundaries (Lupianez et al., 2015). Engineered mice carrying mutations that affect these TAD boundaries, which disturb the topological association between enhancer and promoter elements, prevented the transcriptional activation of genes, and this occurred without mutations in the enhancer nucleotide sequence (Lupianez et al., 2015; Symmons et al., 2016; Tsujimura et al., 2015). Given the unique position of the ZRS (at the edge of the TAD) relative to all other *Shh* enhancers, the ZRS might be more susceptible to inactivation by

mutations that affected the telomeric TAD boundary (Figure 4a). Such mutations could lead to the exclusion of the ZRS from the *Shh* TAD without affecting the location of all other enhancers centromeric to the ZRS (within the TAD). In principle, this hypothetical chromatin-topology mutation of the ZRS would be a potential substrate for limb evolution given its low pleitropic load. A similar phenomenon could underlie the human malformation Acheiropodia (ACHP; OMIM #200500), which causes radical truncation of the limbs. Acheiropodia does not map to the ZRS but to a deletion centromeric to the ZRS, comprising part of intron 3, exon 4, and part of intron 4 of *Lmbr1* (Ianakiev et al., 2001). Further work will be necessary to determine if the deletion in acheiropodia changes chromatin topology in a manner that shifts the ZRS out to the *Shh* TAD and into the contiguous TAD, which would specifically silence *Shh* at the ZPA without pleiotropic effects.

8 | CONSERVATION OF ANCESTRAL DEVELOPMENTAL POTENTIAL: RESCUE OF HINDLIMBS IN SNAKES

One of the most intriguing issues in snake evolution is the phylogenetic position of limbed fossil snakes. The discovery of snake fossils with complete hindlimbs from the Late Cretaceous initially suggested these snakes are basal, and, therefore, reflective of the limbed ancestry of the snake lineage (Caldwell & Lee, 1997). However, discoveries of additional specimens and parallel analyses by multiple investigators led to disagreement over the phylogenetic positions of these limbed snake fossils (Apesteguia & Zaher, 2006; Hsiang et al., 2015; Rieppel, Zaher, Tchernov, & Polcyn, 2003; Tchernov et al., 2000). In particular, derived features of their skulls raised questions about their basal status, instead suggesting that they arose after the vestigalization of hindlimbs (Rieppel et al., 2003; Tchernov et al., 2000). Implicit in the more crownward placement of these snakes is the conclusion that complete legs were reacquired from legless ancestors during snake evolution (Rieppel et al., 2003; Tchernov et al., 2000).

Reemergence of lost morphological characters is a debated issue in evolutionary biology (Collin & Miglietta, 2008; Gould, 1970). Complete limb re-evolution in species derived from limbless ancestors has been proposed for numerous squamate lineages (Brandley et al., 2008; Kohlsdorf & Wagner, 2006). Each of these proposed "reversals" of limb loss is based on phylogenetic analysis of character evolution and ancestral state reconstructions (Brandley et al., 2008; Kohlsdorf & Wagner, 2006); however, lack of data on the genetic or developmental mechanisms that could lead to re-emergence of complete limbs has led some to question the plausibility of such scenarios (Galis, Arntzen, & Lande, 2010). Complete rebuilding of lost structures from a degenerated developmental program seems an unlikely evolutionary phenomenon, and, by extension, this would make re-evolution of complex characters unlikely. However, studies of limb and genitalia development in snakes and lizards indicate that the genetic program for appendage development is surprisingly conserved, even in basal snakes such as pythons, which have radically truncated hindlimbs (Infante et al., 2015; Leal & Cohn, 2015; Tschopp et al., 2014). In python hindlimb buds, the major signaling centers (ZPA, AER, dorsal ectoderm), the gene regulatory

networks (GRNs) that control their activities, and the limb enhancers that govern the activation of these GRNs are extensively conserved, perhaps due to pleiotropic functions in other appendages such as the external genitalia (Infante et al., 2015; Leal & Cohn, 2015; Tschopp et al., 2014). This evidence that the limb developmental program did not degenerate, but instead remained intact for its non-limb related functions, makes re-emergence of legs in snakes a more likely prospect.

Evidence for the conserved function of these circuits has recently come from developmental studies of python leg buds. In addition to genomic evidence that the ZRS was not lost and experimental evidence that its function, while diminished, leads to conserved yet transitory patterns of gene expression in the hindlimb buds, analysis of skeleto-genesis showed that pythons advance to a surprisingly late stages of leg development, forming anlagen of the tibia, fibula, and even the autopod (Leal & Cohn, 2016). Thus, although premature termination of the ZPA–AER feedback loop in pythons causes hindlimb outgrowth to arrest, the early burst of *Shh* expression and AER activity are sufficient to activate late phase expression of the autopodial genes *Hoxd13* and *Hoxa13* (Figure 4c; Leal & Cohn, 2016). Analysis of chondrogenic markers showed that python hindlimb buds have extensive skeleto-genic potential and give rise to all three segments of a tetrapod limb skeleton—stylopod, zeugopod, and autopod—but the distalmost structures degenerate prior to hatching, leaving the mature python with only femoral rudiments (Leal & Cohn, 2016). This observation provides further support to the hypothesis that *Shh* levels in early limb development are important for specification of the limb skeleton, and that sustained *Shh* expression drives expansion of the progenitors and growth of skeletal elements (Shapiro, Hanken, & Rosenthal, 2003; Zhu et al., 2008). As such, the burst of *Shh* expression in python hindlimb buds appears to specify a relatively complete limb (stylopod, zeugopod, and autopod), but failure to sustain *Shh* expression affects the subsequent growth and differentiation limb skeletal progenitor cells.

Snake genomes also contain evidence that the mechanisms of autopod development have been conserved. The genome regulatory landscape for the transcriptional control of Hoxd genes has been characterized in detail during mouse limb development, including the autopodial expression of *Hoxd13* (Montavon et al., 2011). Centromeric to the HoxD cluster lies a large gene desert where a series of "autopodial enhancers" are located (Montavon et al., 2011), and these elements control the expression of posterior *Hoxd* genes (Figure 4c), particularly *Hoxd13*, during the stages of autopod specification and digit morphogenesis (Fromental-Ramain et al., 1996). Analysis of the centromeric gene desert in pythons demonstrated that, although digits were lost deep in the evolutionary history of snakes, autopodial enhancers are still present adjacent to the python HoxD cluster (Leal & Cohn, 2016). Like other genes required for limb development, *Hoxd13* plays a critical role in external genital morphogenesis (Warot, Fromental-Ramain, Fraulob, Chambon, & Dolle, 1997) and it is highly transcribed in the developing hemipenes of python embryos (Leal & Cohn, 2015). The limb and genital expression domains of *Hoxd13* are controlled primarily by the dual centromeric HoxD limb/genital enhancers (with the exclusion of some genital-specific and limb-specific enhancers; Lonfat, Montavon, Darbellay, Gitto, & Duboule, 2014). Therefore, selection against accumulation of

pleiotropic mutations that could affect genitalia morphogenesis also acted to stabilize the developmental program needed for limb development. However, when some of these centromeric enhancers from corn snakes were tested in transgenic mice, it was found that their limb activity has been lost but activity in external genitalia has been preserved (Guerreiro et al., 2016). Given that hindlimb buds in pythons activate an autopodial *Hoxd13* expression domain (Leal & Cohn, 2016), it appears that the limb functions of centromeric HoxD enhancers in pythons have been protected from mutational damage, which contrasts with the results obtained using enhancers of more derived (completely limbless) snakes. These findings lay the groundwork for broader comparisons of enhancer activity across basal and derived snakes.

These new studies raise the possibility that re-acquisition of complete limbs in some (extinct) snake lineages could have resulted from activation of a largely intact but dormant limb developmental program in snake embryos. A simple, yet plausible scenario for re-emergence of limbs in species derived from limbless ancestors is that compensatory mutations in the ZRS could have boosted *Shh* expression by enhancing a hypofunctional ZPA to levels that sustained limb development up to the formation of a complete hindlimbs with toes. Interestingly, this scenario is not far from the experimental rescue of limbs in mice carrying the python ZRS by reversing only one of the python ZRS deletions (Kvon et al., 2016).

9 | CONCLUDING REMARKS

The molecular mechanisms that drove evolution of the snake body plan have been the subject of renewed interest in evolutionary developmental biology. Development of new tools for transgenesis and computational analysis of large data sets is providing unparalleled opportunities to investigate the genetic and genomic mechanisms responsible for the evolution of snakes from four-legged lizard ancestors. In addition, these technological advances should increase the phylogenetic breadth and depth available for study, as investigators are not limited to species from which precious embryonic material can be obtained. An important lesson from recent studies of limblessness in snakes is that comparative genomic approaches are most powerful when combined with functional studies designed to test developmental genetic hypotheses in cells and in embryos. Strategies that leverage and integrate complementary approaches should allow investigators to reproduce actual (and to test hypothetical) evolutionary changes in the genome and to determine the effects of such changes on morphological evolution.

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