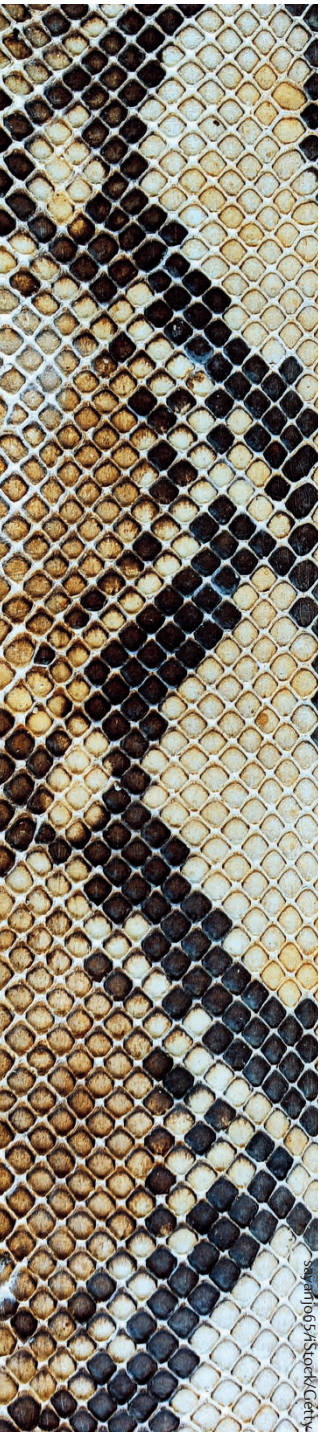
Nature Reviews Genetics | Published online 15 Nov 2016; doi:10.1038/nrg.2016.149

DEVELOPMENT

Sonic snakes and regulation of limb formation



Evolutionary limb loss is a fundamental and overt change in body plan, but the underlying molecular causes have remained debated and largely elusive. Two new studies pinpoint enhancer alterations associated with limb loss during snake evolution.

Mechanisms of major morphological transitions have been challenging to elucidate. The continued presence of known gene families for limb formation in limbless organisms such as snakes, as well as fossil indications that limbless species might retain the capability to re-evolve limbs, suggests that these major macroevolutionary changes may be caused by subtle regulatory alterations.

In their search for mechanistic answers, Kvon *et al.* examined regulation of the Sonic hedgehog (*SHH*) cell-fate gene. They focused on a characterized limb *SHH* enhancer — known as the zone of polarizing activity regulatory sequence (ZRS) — which is known to be essential for proper limb development in several vertebrates. Taking advantage of the recent availability of six draft snake genomes, they compared snake ZRS sequences with those of limbed vertebrates. The ZRS enhancer showed progressive degeneration, relative to limbed vertebrates, in basal snakes (which retain vestigial rudimentary limbs) through to advanced snakes (which have lost all skeletal limb structures). This degeneration did not occur in other limb enhancers examined.

To prove that ZRS sequence differences have functional consequences, the authors tested 16 ZRS sequences from a range of snakes and limbed vertebrates in an *in vivo* mouse enhancer reporter assay. Whereas ZRS enhancers from limbed species drive robust reporter expression in the zone of polarizing activity in developing limbs, this activity is progressively lost in ZRS sequences from basal through to advanced snakes. Furthermore, knock-in to replace a 1.3 kb core region of mouse ZRS with the equivalent sequence from python (a basal snake) resulted in mice with partially truncated limbs, and replacement with the sequence from cobra (an advanced snake) caused substantially more severe limb truncations.

For further mechanistic insight, Kvon *et al.* searched for transcription factor binding motifs that were disrupted by snake-specific deletions, identifying ETS and HOX transcription factors as key candidates. Strikingly, one particular ETS binding site was disrupted by a 17 bp deletion in all snakes, and using CRISPR technology to re-introduce these 17 bp into the python ZRS core sequence of the knock-in mouse restored limb formation, thus highlighting the importance of individual transcription factor binding sites in morphological changes.

In a related study, Leal and Cohn compared SHH signalling in the rudimentary developing limb buds of pythons with those in the limbed green anole lizard. They noted that SHH expression was not sustained in pythons, resulting in a breakdown of the feedback loop and limb development. Hence, they too sought regulatory explanations for disrupted SHH expression in snakes. They found no differences in the expression levels of ZRS-binding transcription factors between the snake and lizard but, similarly to Kvon *et al.*, used comparative genomics to identify progressive degeneration of ZRS sequences during snake evolution and demonstrated functional consequences in mouse reporter assays.

Leal and Cohn identified three deletions at the 5′ end of the ZRS that occur in snakes but not limbed vertebrates; the middle deletion is the same 17 bp deletion studied by Kvon *et al.* They showed using engineered expression constructs in cell culture that each deletion reduces enhancer activity (although effects on limb development *in vivo* were not explicitly tested). Although Leal and Cohn note loss of an ETS binding site caused by the 17 bp deletion, their co-expression experiments and electromobility shift assays indicated that each of the three identified ZRS deletions disrupt binding by HOXD homeodomain transcription factors, as a potential molecular explanation for curtailed SHH signalling and limb development in snakes.

It will be interesting to dissect the relative importance of the evolutionary binding site disruptions identified by Kvon *et al.* and Leal and Cohn, as these sites seem to function in a redundant manner. For example, limb formation can be rescued by restoring a single 17 bp deletion, as shown by Kvon *et al.* It remains to be seen whether restoring this deletion could over-ride ZRS enhancer deficiency in the context of other binding site deletions, as well as the relative importance of ETS versus HOXD binding events for limb loss and re-establishment.

Finally, knowledge of the identified regulatory mechanisms may help the clinical interpretation of non-coding mutations in human patients with limb malformations.

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ORIGINAL ARTICLES Kvon, E. Z. *et al.* Progressive loss of function in a limb enhancer during snake evolution. *Cell* **167**, 633–642 (2016) | Leal, F. & Cohn, M. J. Loss and re-emergence of legs in snakes by modular evolution of sonic hedgehog and *HOXD* enhancers. *Curr. Biol.* http://dx.doi.org/10.1016/j.cub.2016.09.020 (2016)