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# A relative shift in cloacal location repositions external genitalia in amniote evolution

Patrick Tschopp, Emma Sherratt, Thomas J. Sanger, Anna C. Groner, Ariel C. Aspiras, Jimmy K. Hu, Olivier Pourquié, Jérôme Gros &amp; Clifford J. Tabin

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The move of vertebrates to a terrestrial lifestyle required major adaptations in their locomotory apparatus and reproductive organs. While the fin-to-limb transition has received considerable attention<sup>1, 2</sup>, little is known about the developmental and evolutionary origins of external genitalia. Similarities in gene expression have been interpreted as a potential evolutionary link between the limb and genitals<sup>3, 4, 5, 6</sup>; however, no underlying developmental mechanism has been identified. We re-examined this question using micro-computed tomography, lineage tracing in three amniote clades, and RNA-sequencing-based transcriptional profiling. Here we show that the developmental origin of external genitalia has shifted through evolution, and in some taxa limbs and genitals share a common primordium. In squamates, the genitalia develop directly from the budding hindlimbs, or the remnants thereof, whereas in mice the genital tubercle originates from the ventral and tail bud mesenchyme. The recruitment of different cell populations for genital outgrowth follows a change in the relative position of the cloaca, the genitalia organizing centre. Ectopic grafting of the cloaca demonstrates the conserved ability of different mesenchymal cells to respond to these genitalia-inducing signals. Our results support a limb-like developmental origin of external genitalia as the ancestral condition. Moreover, they suggest that a change in the relative position of the cloacal signalling centre during evolution has led to an altered developmental route for external genitalia in mammals, while preserving parts of the ancestral limb molecular circuitry owing to a common evolutionary origin.

**Subject terms:** Evolutionary developmental biology Cell lineage Transcriptomics

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## Accession codes

### Primary accessions

Gene Expression Omnibus

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## Author information

**Present addresses:** Department of Ecology, Evolution, and Organismal Biology, Iowa State University, Ames, Iowa 50011, USA (E.S.); Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida 32610, USA (T.J.S.); Department of Orofacial Sciences and Program in Craniofacial and Mesenchymal Biology, UCSF, San Francisco, California 94143, USA (J.K.H.).

Emma Sherratt, Thomas J. Sanger & Jimmy K. Hu

### Affiliations

**Department of Genetics, Harvard Medical School, Boston, Massachusetts 02115, USA**

Patrick Tschopp, Ariel C. Aspiras, Jimmy K. Hu, Olivier Pourquié & Clifford J. Tabin

**Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts 02138, USA**

Emma Sherratt & Thomas J. Sanger

**Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, USA**

Anna C. Groner

**Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), 67400 Illkirch, France**

Olivier Pourquié

**Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA**

Olivier Pourquié

**Developmental and Stem Cell Biology Department, Institut Pasteur, 75724 Paris Cedex 15, France**

Jérôme Gros

### Contributions

P.T., J.G. and C.J.T. conceived the project and designed the experiments. P.T. performed most experiments and computational analyses. E.S. prepared CT scans and helped with statistical analyses. T.J.S. helped with CT scans, *Anolis* husbandry and embryo collection. A.C.G. produced lentiviruses and A.C.A. helped with grafting experiments. J.K.H., O.P. and J.G. initiated snake analyses. O.P. contributed snake embryos. J.G. contributed to chick lineage tracing experiments. P.T., J.G. and C.J.T. wrote the paper, with comments from co-authors.

### Competing financial interests

The authors declare no competing financial interests.

### Corresponding authors

Correspondence to: Clifford J. Tabin or Jérôme Gros

Sequencing data has been deposited in the Gene Expression Omnibus under accession number GSE60373.

## Extended data figures and tables

### Extended Data Figures

1. Extended Data Figure 1: Two separable ventral cell populations give rise to the murine genital tubercle. (332 KB)

**a, b**, Injection into the most distal ventral part of the embryo, the tail bud, marks cells posterior/ventral to the phallic part of the urethra (**a**, arrow;  $n = 7$ ), whereas injection closer to the allantois, into the infra-umbilical mesenchyme, labels cells anterior/dorsal to the phallic part of the urethra (**b**, arrow;  $n = 4$ ). Cells lining the peritoneal cavity are also marked (arrowheads), owing to accidental piercing of the coelom. **gt**, genital tubercle; **ur**, urethra. Scale bars, 200  $\mu\text{m}$ .

2. Extended Data Figure 2: The squamate hemipenis mesenchyme initiates with limb-like cellular dynamics from the coelomic epithelium through an EMT. (1,750 KB)
 

**a**, Injection of GFP-expressing lentiviruses into the coelom of chicken embryos at HH14 labels cells emerging from the epithelium that contribute to the hindlimb mesenchyme (arrowhead). **b**, In lizards, labelled cells leaving the coelomic epithelium contribute to the hemipenis mesenchyme (arrowheads). **c**, Dorsal view of the hindlimb region of an E10.0 mouse embryo. **d**, Transversal section of a limb bud, showing EMT of the coelomic epithelium (diffuse laminin staining, open arrowhead), as cells contribute to the limb-bud mesenchyme. **e**, Dorsal view of the budding hemipenis of a snake embryo, 1 day after egg deposition. **f**, Transversal section of the hemipenis region. The basement membrane of the coelomic epithelium is breaking down (open arrowhead), while it is intact for both the nephric duct and the surface ectoderm (arrowheads). **g–o**, Expression of genitalia and limb genes during hemipenis initiation. **g–i**, *Tbx4* is expressed early (**h**, arrow) and late during hemipenis initiation, in both the coelomic epithelium (**i**, arrowhead) and the hemipenis mesenchyme (**i**, arrow). **j–l**, *Tbx5* is only expressed later, in the mesenchyme (**l**, arrow), but is absent from the coelomic epithelium (**l**, open arrowhead). **m–o**, Limb marker gene *Lhx9* (see also Fig. 4e) is absent from both epithelium (**o**, open arrowhead) and mesenchyme (**o**, open arrow), but can be detected in dl1 neurons (**o**, asterisk). All gene expression was assessed in at least  $n = 3$  samples. **cl**, cloaca; **co**, coelom; **hp**, hemipenis; **lb**, limb; **nd**, nephric duct. Scale bars, 50  $\mu\text{m}$ .
3. Extended Data Figure 3: Heat maps of Pearson's and Spearman's rank correlation coefficients and cluster analysis of whole-transcriptome data. (293 KB)
 

**a, b**, Hierarchical clustering on pairwise correlation coefficients for whole-transcriptome data from anole (**a**) and mouse (**b**) samples. Numbers at nodes represent approximately unbiased  $P$  values obtained by multiscale bootstrap resampling. Sample identifiers: **a**, anole; **m**, mouse; **GT**, genital tubercle; **HP**, hemipenis; **LB**, limb; **e**, early; **l**, late.
4. Extended Data Figure 4: Heat maps of Pearson's and Spearman's rank correlation coefficients and cluster analysis of transcription factor and signalling pathway data. (292 KB)
 

**a, b**, Hierarchical clustering on pairwise correlation coefficients of transcription factor (TF) and signalling pathway data from anole (**a**) and mouse (**b**) samples. Numbers at nodes represent approximately unbiased  $P$  values obtained by multiscale bootstrap resampling. Sample identifiers: **a**, anole; **m**, mouse; **GT**, genital tubercle; **HP**, hemipenis; **LB**, limb; **e**, early; **l**, late.
5. Extended Data Figure 5: Heterotopic grafting of the cloacal signalling centre leads to ectopic outgrowths and genitalia-like transcriptional changes. (867 KB)
 

**a–c**, Schematics and close-up images of the cloacal grafting procedure. **a**, The cloaca of a stage HH17–19 GFP-transgenic chicken embryo (red rectangle) is transplanted into the proximal-ventral portion of the limb of a wild-type embryo. **b, c**, Only the ventral-most part of the cloaca, including the cloacal membrane, is dissected out (**b**, red box), and subsequently cleared of excess mesenchymal cells attached to the *SHH*-expressing endoderm (**c**, red outline). **d–g**, Grafting of beads soaked in SHH and FGF can induce ectopic outgrowths on both limbs (**e**;  $n = 6/48$ ) and tail (**g**;  $n = 3/31$ ). **h–k**, Ectopic expression of genital markers *GATA2* (**h, i**, arrowheads) and *RUNX1* (**j, k**, arrowhead) in limb buds, following cloaca-to-limb grafts. **l–n**, Ectopic expression of genital marker *GATA2* (**m**, arrowheads) and *RUNX1* (**n**, arrowheads) in the tail region, following cloaca-to-tail grafts. All gene expression was assessed in at least  $n = 3$  samples. **al**, allantois; **cl**, cloaca; **lb**, limb. Scale bar, 200  $\mu\text{m}$ .
6. Extended Data Figure 6: Pairwise differential expression analysis of limb and genitalia transcriptomes. (404 KB)
 

**a–d**, Smear plot visualization of differential expression analyses of early anole (**a**), late anole (**b**), early mouse (**c**) and late mouse (**d**) limb versus genitalia transcriptomes. Genes used for the Venn diagram in Fig. 4d ( $|\log_2(\text{fold change})| > 1.5$ ;  $P$  value  $< 0.05$ ) are highlighted in red, core 25 marker genes (see Fig. 4e and text) are highlighted and labelled in blue. CPM, counts per million; FC, fold change. **e, f**, Heat map of Z-score-normalized expression values for all genes fulfilling Venn diagram criteria ( $n = 2,003$ ), for anole (**e**) and mouse (**f**) data. Row-based hierarchical clustering was used; core 25 marker genes are indicated on the right.
7. Extended Data Figure 7: Comparative marker gene expression analysis in mouse and squamate embryos. (615 KB)
 

**a–r**, Genitalia markers *Isl1* (**a–d**), *Runx1* (**e, f**), *Gata2* (**g–j**), *Eya4* (**k, l**), *Tbx5* (**m–p**) and *Dkk2* (**q, r**). *Gata2* only becomes visibly expressed at the later stages of house snake hemipenis development (**j**, inset). **s–a'**, Limb markers *Lhx9* (**s–v**), *Tbx18*

(**w**, **x**) and *Lmx1b* (**y–a'**). All gene expression was assessed in at least  $n = 3$  samples. cl: cloaca; gt: genital tubercle; hp: hemipenis; lb: limb. Scale bar, 200  $\mu\text{m}$ . [More info.](#)

8. Extended Data Figure 8: Pairwise differential expression analysis of tail bud and genitalia transcriptomes. (219 KB)  
**a**, **b**, Smear plot visualization of differential expression analyses of early anole (**a**) and early mouse (**b**) tail bud versus genitalia transcriptomes. Genes used as input for the Venn diagram in **c** ( $|\log_2(\text{fold change})| > 1.5$ ;  $P$  value  $< 0.05$ ) are highlighted in red, overlapping 257 marker genes are highlighted in blue. Top 25 genes in the two species, based on logCPM (counts per million) and logFC (fold change), are labelled. **c**, Venn diagram showing overlap of pairwise differential expression analysis results ( $\log(\text{fold change}) > 1.5$ ,  $P$  value  $< 0.05$ ) of tail bud versus genital tissues for early budding stages in both anole and mouse.

## Supplementary information

### PDF files

#### 1. Supplementary Table 1 (3.6 MB)

This file contains a list of orthologous genes between mouse and anole employed for GO-term analysis. Genes are ordered according to their absolute loading value from principal component analysis (see Fig. 3e), for principal component 2 (PC2), and top 500 genes (in bold, see Fig. 3f) were used for GO-term enrichment analysis.

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