The vertebral column of individual mammalian species often exhibits remarkable robustness in the number and identity of vertebral elements that form (known as axial formulae). The genetic mechanism(s) underlying this constraint however remain ill-defined. Here, we reveal the interplay of three regulatory pathways (Gdf11, miR-196 and Retinoic acid) is essential in constraining total vertebral number and regional axial identity in the mouse, from cervical through to tail vertebrae. All three pathways have differing control over Hox cluster expression, with heterochronic and quantitative changes found to parallel changes in axial identity. However, our work reveals an additional role for Hox genes in supporting axial elongation within the tail region, providing important support for an emerging view that mammalian Hox function is not limited to imparting positional identity as the mammalian body plan is laid down. More broadly, this work provides a molecular framework to interrogate mechanisms of evolutionary change and congenital anomalies of the vertebral column.
The mammalian vertebral column is comprised of serially-repeating vertebrae that, while individually-identifiable, are grouped based on morphological and functional similarity into cervical (C), thoracic (T), lumbar (L), sacral (S) and caudal (Ca) elements. Vertebrae arise from developmentally-transient structures called somites, that are in turn generated from a progenitor pool located in the posterior embryo through the coordinated processes of axial elongation and segmentation. The genetic mechanisms dictating vertebral identity are well understood, and centre on the temporally-controlled activation of Hox cluster genes within vertebral progenitors. In contrast, uncovering the genetic mechanisms that control vertebral number of each axial region in a meristic rather than homeotic manner (as defined in Bateson, 1894), and consequently total vertebral number (TVN) of a mammalian species, has proved incredibly challenging.

The changes that constitute diversity of mammalian axial formulae are not uniform along the anterior-posterior (A-P; head-to-tail) axis. Cervical number is almost exclusively fixed at 7, a trait that has rarely changed over 200 million years of mammalian evolution, spanning back to the mammalian ancestor of the late Triassic. Two rare exceptions to this rule, manatee and sloth, have sparked intense interest in the advantage and developmental basis of these breaks in constraint, with their genetic basis unresolved to date. Thoraco-lumbar (T-L) count, while less rigid, is still highly conserved at 19 or 20 across most mammals, though again with examples of relaxation in both absolute T-L number and intraspecies robustness seen in clades such as Xenarthra and Afrotheria. Sacral count varies considerably across mammals and along with the extreme diversity seen in caudal/tail morphology reinforces a graded decrease in constraint along the A-P axis.

As mouse displays the likely ancestral mammalian presacral formulae, Rodentia exhibits the lowest deviation from median vertebral counts of all mammals, it should serve as an excellent model with which to dissect genetic mechanisms constraining axial formulae. However, only recently a truncating mouse mutants exist, often being highly dysmorphic, the ability to increase TVN in a meristic not simply homeotic manner has been limited to perturbation of only two genetic pathways (see Supp Information for note on Hox13 mouse mutant phenotypes). The largest of these two meristic increases was observed in, perhaps intuitively, the evolutionary more plastic head-to-tail (A-P) axis.

To determine whether the combined function of Gdf11 and miR-196 identified in vitro influences axial elongation in vivo, we interbred miR-196-TKO and Gdf11+/− mice and characterised TVN and vertebral identity across the allelic deletion series (Fig. 2a–d, Supp. Table 1). Consistent with previous reports, complete loss of miR-196 alone resulted in an increase of 2 thoracic elements (C7, T22, L10; Fig. 2b), while complete loss of Gdf11 alone resulted in a dramatically expanded presacral region (C7, T15, L6, median TVN 66). Here, we show that in vitro modelling of axial progenitors identifies unexpected cooperation between transcriptional and post-transcriptional regulatory mechanisms that act to terminate presacral expression signatures. Applying this knowledge in vivo, we are able to experimentally prolong the natural process of axial elongation in the mouse and considerably increase total vertebral number within both presacral and caudal regions. We reveal a high level of redundancy and synergism between Gdf11, miR-196 and retinoic acid (RA) in the constraint of axial formulae, with Gdf11 and RA cooperativity impacting a surprisingly large extent of the vertebral column including the constraint of cervical number to 7. All regulatory mechanisms converge in their ability to control spatio-temporal Hox expression, a parameter that our data supports having a substantive role in axial elongation, at least in the tail region.

**Results**

**Synergistic constraint of trunk Hox expression signatures.** To dissect mechanisms constraining regionally-restricted Hox expression, as a proxy for regional axial morphology, we initially focused on modelling the trunk-to-tail (T-T) transition in vitro since progression through this critical moment in body axis formation correlates with the switch from an expanding to a depleting and eventually exhausted progenitor pool in species as diverse as mouse and snake. miR-196 has a non-redundant role in timing the T-T transition, and thus we generated induced pluripotent stem cells (iPSCs) from wild-type (WT) and miR-196aΔfl/fl, miR-196aΔfl/fl; miR-196b−/− (miR-196-TKO) isogenic mice using standard protocols (Supp. Figure 1a). Both iPSC lines were differentiated to model the developmental kinetics of a posterior growth zone additional of FGF2 on Day (D) 0 and the Wnt pathway agonist CHIR99021 on D2 (Fig. 1a). Consistent with previous work, miR-196-TKO iPSCs were able to generate axial progenitors normally, however, exhibited marked differences in the kinetics of Hox cluster progression when compared to WT (Fig. 1b). Trunk Hox genes Hoxb8 and Hoxc8 displayed an identical timing of activation for both genotypes, but as differentiation proceeded, their quantitative level and temporal persistence were significantly increased in miR-196-TKO cells (Fig. 1b), consistent with the presence of functional miR-196 binding site(s) within their 3′UTRs. Addition of Gdf11 to culture conditions was able to suppress trunk Hox gene expression and induce posterior Hox activation in both genotypes as anticipated. However, in the absence of Gdf11, both Hoxb8 and Hoxc8 displayed an unrestrained upwards trajectory in miR-196-TKO cells but not in WT (Fig. 1b). This key result indicated that miR-196 and Gdf11 are not simply redundant in this context, but act in concert in shutting down a trunk Hox code, a parameter suggested to support axial elongation under certain circumstances.

Gdf11 and miR-196 synergistically constrain TVN. To determine whether the combined function of Gdf11 and miR-196 identified in vitro influences axial elongation in vivo, we interbred miR-196-TKO and Gdf11+/− mice and characterised TVN and vertebral identity across the allelic deletion series (Fig. 2a–d, Supp. Table 1). Consistent with previous reports, complete loss of miR-196 alone resulted in an increase of 2 thoracic elements (C7, T15, L5, median TVN 62), while complete loss of Gdf11 alone resulted in a dramatically expanded presacral region (C7, T18, L5, median TVN 66). As mouse displays the likely ancestral mammalian presacral formulae (see Supp Information for note on Hox13 mouse truncation phenotype, we found that Gdf11+/−/−TKO cells but not in WT (Fig. 1b). This key result indicated that miR-196 and Gdf11 are not simply redundant in this context, but act in concert in shutting down a trunk Hox code, a parameter suggested to support axial elongation under certain circumstances.
date. Together, these data provide 3 major advances: (i) Gdf11 activity is required under wild-type conditions to constrain total vertebral number; (ii) the vertebral column is exquisitely sensitive to dosage of Gdf11, with opposing outcomes revealed dependent on allele number; (iii) miR-196 and Gdf11 do not constitute entirely parallel mechanisms in the control of presacral vertebral number but rather, act synergistically, since the effect of their combined loss is greater than the sum of individual mutant phenotypes (Fig. 2d).

Retinoic acid has pleiotropic effects on axial formulae. While TVN cannot be accurately quantified in Gdf11\textsuperscript{−/−} embryos due to the highly dysmorphic nature of post-sacral elements, the loss of miR-196 activity did not qualitatively appear to rescue Gdf11\textsuperscript{−/−} truncation (Fig. 2a). In this context, the partial rescue of Gdf11\textsuperscript{−/−} truncation by reducing endogenous levels of Retinoic acid (RA) signalling\textsuperscript{31} led us to examine the potential for more elaborate redundancy or synergy between the three signalling/regulatory pathways. Indeed, oral gavage of pregnant dams across the genetic deletion series with pan-RA receptor inhibitor AGN193109 (AGN) revealed further combinatorial changes in vertebral number and identity, spanning each major subdivision of the vertebral column (Fig. 3a–c; Supp. Figure 2a; Supp. Table 2). Notably, we showed that RA constrains TVN in mouse, with an additional 1–2 elements observed following RA depletion for all genotypes where TVN can be quantified, including WT, Gdf11\textsuperscript{−/−}, miR-196-TKO and Gdf11\textsuperscript{−/−};miR-196-TKO embryos. This increase manifested as an expansion in the number of presacral elements, the identity of which (see ‘Methods’) was dependent on the exact allelic combination (Compare Supp. Figure 2a with Fig. 2a). AGN-exposed WT embryos yielded anteriorising transformations of the cervical region (C2 \rightarrow C1, C7 \rightarrow C6) at low penetrance, no change in positioning of cervico-thoracic transition (Fig. 3c) and one additional T element (Supp. Figure 2a). All phenotypes were consistent with observed phenotypes in Retinoic acid receptor γ (RARγ) knockout mice\textsuperscript{32}. The deletion of even a single Gdf11 allele under these RA-depletion conditions lead to a higher penetrance of C7 \rightarrow C6 transformation, and in 40% of embryos, serial transformation resulted in a shift in cervico-thoracic positioning such that 8 cervical elements formed (Fig. 3c). This striking shift in cervico-thoracic positioning, widely considered as one of the strongest evolutionarily constrained traits of mammals\textsuperscript{6,33}, became almost fully penetrant in Gdf11\textsuperscript{−/−} embryos treated with AGN, and where present, usually formed at the expense of a thoracic element and thus was homoeotic in nature. Vertebral identity at the cervico-thoracic junction is known to involve the action of Hox5/6 paralogous groups\textsuperscript{34} early in development. Consistent with this, and with observed phenotypic changes, we found the anterior boundary of Hox6 was shifted caudally by 1 somite in E10.5 Gdf11\textsuperscript{−/−} mutants treated with AGN (Supp. Figure 2b), demonstrating redundancy between RA and Gdf11 in the timely activation/spatial regulation of Hox6, and likely other trunk Hox genes. Countering this displacement of the cervico-thoracic junction, deletion of miR-196 paralogs returned this major morphological transition back to normal (Fig. 3d, Supp. Table 2), likely through the relief of post-transcriptional suppression of Hox5/6 targets\textsuperscript{16,35} at this site. Nonetheless, additional presacral element(s) still formed in AGN-treated miR-196-TKO, Gdf11\textsuperscript{−/−};miR-196-TKO and Gdf11\textsuperscript{−/−};miR-196-TKO embryos, taking on a thoracic or lumbar identity (Compare Fig. 2d with Fig. 3d; Supp. Table 2). Collectively, this extensive genetic/chemical deletion series has revealed that RA, Gdf11 and miR-196 all act to constrain TVN individually, and synergistically. In addition, each factor differentially shapes positional identity along the A-P axis, with individual, synergistic and, in this case, antagonistic interactions revealed. It is important to note that in these embryos, and all embryos assessed in this study, progression through the T-to-T transition always occurred, albeit late. This indicates that the regulatory synergism promoting this key transition was yet to be fully depleted, with prime candidates supporting the eventual T-to-T in compound mutant embryos being Gdf8\textsuperscript{36} and potentially FGF signalling\textsuperscript{37}.

Factors constraining TVN differentially shape Hox codes. One shared feature of the extrinsic and intrinsic mechanisms shown here to constrain TVN and shape regional identity is their capacity to influence spatio-temporal Hox expression\textsuperscript{16,17,38}. Thus, to reveal the full extent to which miR-196 and Gdf11 individually and collectively regulate Hox codes during the T-to-T transition (somites 21 \rightarrow 32 [-E9.5]) or after this transition is complete (somite 32 \rightarrow [-E10.5]), we quantified expression of all 39 Hox genes within tailbud tissue across the allelic deletion series (Fig. 4). Removal of miR-196 repressive activity (miR-196a\textsuperscript{−/−};b\textsuperscript{−/−}, phenotypically equivalent to miR-196-TKO) led to a robust upregulation of trunk Hox genes and concomitant downregulation, or failure to timely activate, posterior Hox genes.
of all four clusters at E9.5 (Fig. 4)\textsuperscript{16}, a signature that began to resolve by E10.5 (Fig. 4). From a patterning perspective, either an increased trunk Hox code\textsuperscript{39} or a delay in activation of Hox10 rib-suppression activity\textsuperscript{3,40} have the potential to drive the expanded thoracic identity seen in \textit{miR-196} TKO embryos. In contrast, loss of one \textit{Gdf11} allele had minimal impact on trunk Hox expression, indicating that the mildly expanded thoracic phenotype of \textit{Gdf11}\textsuperscript{-/-} embryos is more likely to result from the modest heterochronic delay in posterior Hox code activation seen at E9.5, which largely resolved to WT levels by E10.5 (Fig. 4). This is further supported in \textit{Gdf11} TKO embryos, where the level of trunk Hox upregulation was not consistent with the dramatically expanded thoracic phenotype, however, a striking reduction in expression of all posterior Hox genes was seen at E9.5 (Fig. 4).
Finally, the altered molecular signature revealed following combined loss of all miR-196 and Gdf11 alleles reflects the synergistic morphological outcomes observed in presacral regions (Fig. 2a; Supp. Table 1). Collectively, the altered Hox codes observed across the allelic deletion series allow us to rationalise the molecular basis for observed patterning changes (Fig. 2d; Supp. Table 1). Moreover, the ability for ectopically-expressed trunk Hox genes Hoxa5 or Hoxb8 to drive axial elongation under certain circumstances and the suggested importance of timely activation of Hox13 paralogs in terminating mouse axial elongation, raised the question as to whether the hetero-
chronic and quantitative changes in global Hox signatures observed here may also underlie changes in TVN.

**Posterior Hox expression supports tail formation.** The understanding of Hox12 and Hox13 paralog function during vertebral column formation is incomplete at best due to the current lack of paralog group mouse knockouts, and recently, the view of Hox13
genes as endogenous axis terminators has been challenged in fish. To interrogate posterior Hox function, and specifically, to dissect whether the delay in/loss of posterior Hox activation seen in Gdf11+/− and Gdf11−/− mutant embryos is of any phenotypic consequence, we sought to restore timely posterior Hox expression through the transgenic mixing of mice expressing either Hoxd11 (Hoxd11OE) or Hoxd12 (Hoxd12OE) under the control of Cdx2 regulatory elements.

Initial characterisation of these lines on an otherwise WT background revealed a reduction in lumbar count by 1 element and concomitant reduction in TVN, with both qualitative (Hoxd12 > Hoxd11) and quantitative (copy number) enhancement of phenotype observed (Fig. 5a–c; Supp. Figure 4c–d; Supp. Table 3). This very mild reduction phenotype of the presacral region was surprising when compared with full tail truncation observed following ectopic expression of Hox13 paralogs which may stem from quantitative differences between transgens or likely functional differences between these Hox proteins. A near-identical reduction in lumbar count/TVN was observed when either Hoxd11OE or Hoxd12OE was bred onto a Gdf11+/− background (Fig. 5a–c; Supp. Figure 4c,e; Supp. Table 3), supporting the view that the temporary delay in posterior Hox activation in these heterozygous embryos (Fig. 4) may indeed contribute to a shifted T-to-T transition and elongation phenotype, or at a minimum, that ectopic posterior Hox expression is dominant over elongation mechanisms.

Each of the above genetic crosses represents a cumulative Hox scenario that is ectopic over WT levels, and thus we next cross-bred either Hoxd11OE or Hoxd12OE onto the Gdf11−/− background, which is greatly depleted for posterior Hox expression (Fig. 4). Relative to truncated Gdf11−/− embryos, restoration of a single posterior Hox gene yielded striking restoration of a segmented Uncx4.1+ tail-like structure at E12.5 and E13.5, albeit ventrally displaced (Fig. 6a; Supp. Figure 5a-b). Detailed characterisation of Gdf11−/− embryos via tissue sections (Supp. Figure 6a) revealed in many cases that the notochord underlying the primary neural tube had turned abnormally relative to WT, projecting ventrally at the level of the cloaca. There, notochordal cells were seen interspersed with Sox2+/−-neural and enveloped by Foxa2+/−-endodermal cells, with no observable patterning or structure to this previously noted ventral mass (Supp. Figure 6a [v–vii]). The restoration of either Hoxd11 or Hoxd12 expression in Gdf11−/− embryos did not prevent ventral projection of the notochord, however, these cells no longer became trapped and extended to the tip of the ventral tail in an organised manner (Fig. 6b, Supp. Figure 6a [ix–xii], [Supplementary movies 1-3]). Adjacent to the notochord in these Gdf11−/−;Hoxd1112OE embryos, an organised Foxa2+/−-tailgut formed, extending from the cloacal orifice to the tip of the ventral tail, with distal cells co-expressing Foxa2 and Sox2 (Fig. 6b, Supp. Figure 6b, [Supplementary movies 1-3]).

Discussion

Historical hereditary studies have long supported a multigenic contribution to even mild examples of intraspecies vertebral variation. Here, we reveal the highly integrated manner by which multiple regulatory layers constrain the modular logic of the vertebral column. Importantly, we were able to produce viable offspring with 5 additional vertebral elements spanning both trunk and tail regions, and with a maximal expansion of 7 additional elements. Regarding the latter, we expect that spatial and/or temporal conditional deletion of these regulatory mechanisms would circumvent embryonic lethality, enabling a vastly altered mammalian body plan, though the potential secondary consequences of this manipulation on locomotion or internal organ formation cannot be predicted. Combined with the recent identification of the Lin28/let-7 axis in constraining vertebral number in the tail, these results demonstrate the depth of genetic redundancy that normally acts to constrain each vertebral region in the mouse and provide robustness to the system as a whole.

The comprehensive allelic deletion series performed allowed us to investigate how the breaking of regional constraint within this experimental model aligns with current hypotheses of natural variation. For example, the break in C7 constraint observed in sloths has been suggested to result from a mis-alignment of somite-derived (primaxial) vertebral elements with that of lateral plate mesoderm-derived (abaxial) distal rib/sternum, such that 7
**Fig. 5 Qualitative and quantitative Hox functions in the control of lumbar count and total vertebral number.**

**a-c** Transgenic expression of Hoxd11 or Hoxd12 using the Cdx2 promoter is able to partially reverse anterior transformations of the lumbar region in Gdf11+/− and Gdf11−/− mutant mice, and reduce total vertebral number in WT and Gdf11+/− animals. **a** E18.5 skeletal analysis of Hoxd11OE or Hoxd12OE (OE = overexpressor) intercrossed with the Gdf11 mutant line revealed changes in axial formulae. C = cervical; T = thoracic; L = lumbar. **b** Quantification of total vertebral number (TVN) in WT and Gdf11+/− embryos, with or without the presence of Hoxd11OE and Hoxd12OE transgenes. Raw data is presented in the upper plot (vertical error bar = mean and standard deviation). Mean differences relative to WT are presented in the lower plot as bootstrap sampling distributions. Each mean difference is depicted as a dot and 95% confidence interval is indicated by the ends of the vertical error bar. n refers to the number of individual animals used for this analysis. Source data for **b** are provided as a Source data file. **c** Schematic summary of changes in axial formulae, relative to WT, observed in single Hoxd11OE or Hoxd12OE transgenic lines, and following cross-breeding of each transgenic with the Gdf11 mutant line. Numbers represent the rounded (to the next whole number) unpaired mean difference for a given genotype. C = cervical, T = thoracic, L = lumbar, S-Ca = sacral-caudal, question marks indicate dysmorphic and non-quantifiable elements. White drawn line = frequently observed reduction/malformation of ribs on the last rib-bearing thoracic element in mice carrying the Hoxd11OE or Hoxd12OE transgene.
cervical vertebral bodies always form and axial diversity arises due to the gain or loss of ribs. Here, in RA-depleted Gdf11−/− embryos with 8 cervical elements, we observe serial anteriorising homeotic transformations for at least 2 elements on either side of the C-T boundary supporting a genuine increase in cervical number. However, in the majority of RA-depleted Gdf11+/− embryos where 7 cervical elements formed, we see that the pattern of centra ossification of the first rib-bearing element is more indicative of a cervical element, and T2 is displaced posteriorly. This suggests that loss of even one Gdf11 allele in this context has the ability to preferentially repattern primaxial tissue, and supports the mis-alignment of primaxial and abaxial tissues as a mechanism to maintain constraint (here) or drive diversity (in sloths).

At a molecular level, the 3′-to-5′ sequential activation of Hox cluster expression within axial progenitors over development time—the Hox clock—prefriges Hox spatial linearity along the A-P axis, though the consequences of clock manipulation are not easy to address. The mouse mutants presented here, all unified in their temporal control of global Hox transitions, offer a granular view as to the consequences of manipulating that Hox clock in vivo. We show that the speeding up, or the slowing down, of Hox cluster signatures result for the most part in serial homeotic transformations that associate with changes in total vertebral number. At a minimum, this implies a very tight association between Hox patterning and elongation mechanisms, though importantly, our data also revealed that posterior Hox genes have the capacity to positively influence axial elongation. This latter data, viewed along with similar results for trunk Hox genes in mouse and Hox13 paralogs in zebrafish, allows us to propose a model whereby multiple post-occipital expressing Hox paralogs participate in construction of the main body axis, not solely in its patterning. We do not suggest that Hox genes are the primary drivers of axial elongation, but that a minimum level is required. This model does not preclude a role for high levels of posterior Hox expression in terminating axial elongation, and indeed our data on ectopic Hoxd11/Hoxd12 can be viewed as supporting this. However, caution should be taken when interpreting overexpression studies while awaiting the genetic deletion of Hox12 and Hox13 paralog groups in the mouse. Why then has a role for Hox genes in shaping vertebral number not been apparent in the extensive Hox mouse mutant literature? Cumulative mutant analysis has shown that each individual vertebral element is patterned by at least two, if not more, Hox paralogs groups, thus only by removal of multiple adjacent paralog groups would this function be revealed. Experimentally, this would be a complex undertaking and of questionable relevance from an evolutionary perspective. However, by altering the timing of collective Hox code transitions (i.e. in vivo pacing of the Hox clock) rather than Hox function per se, through changes in widespread post-transcriptional regulation (miR-196) or alterations in higher-order signalling (Gdf11 haploinsufficiency), this unanticipated vertebrate Hox function has now been revealed.

From a cellular perspective, we show here the persistence of axial progenitors in the tailbud of Gdf11−/−;Hoxd12OE embryos beyond what is seen in WT. Whether the restoration of posterior Hox expression in this scenario is acting cell-autonomously to maintain progenitor proliferation and support tail formation remains to be elucidated, though recent work in zebrafish argues against such a role. Serial transplantation of axial progenitors in vivo has shown that these cells do not intrinsically exhaust following a timer mechanism, highlighting the importance of extrinsic signals in the maintenance and exhaustion of a progenitor pool. In this light, the restoration of tailbud formation along the full extent of the Gdf11−/−;Hoxd12OE ventral tail was of particular interest, since surgical removal of the caudal endoderm in chick has been shown to redirect the notochord ventrally into the hindgut, with striking similarity to what is seen in Gdf11−/− mutants. Moreover, expression of a dominant-negative Hoxa13 protein within chick caudal endoderm, but not mesoderm, resulted in tail truncation, further supporting the positive requirement for posterior Hox function in tail construction across multiple species, and highlighting important cellular targets for future investigation.

By focusing on how one mammalian species cannalises axial formulae, this work has reshaped the current understanding of...
body plan formation and the critical function of Hox networks within. This should help inform the basis for supernumerary vertebral elements that have been reported to appear in ~3% of the human population\textsuperscript{66}, constituting congenital anomalies such as an 8th cervical vertebra\textsuperscript{25},\textsuperscript{27}, additions of up to 3 thoraco-lumbar vertebrae\textsuperscript{58,60} and rare cases of humans with tail vertebrae\textsuperscript{31}. Moreover, the comprehensive allelic deletion series performed here provides us with a window into how evolutionary changes may have occurred, not by complete ablation of one signal or regulatory region, but by more subtle and/or tissue-restricted changes in multiple pathways that converge in their ability to shape axial form.

Methods

Animal experimentation and ethical approvals: All animal procedures were performed in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (2013). Experiments were approved by the Monash Animal Ethics Committee under project numbers MARP/2011/012, MARP/2015/168 and MARP/2015/123.

Mice. mir-196a1GFP, mir-196a2GFP, mir-196a1+/−, mir-196a2+/− and mir-196b−/− mouse lines have been previously described\textsuperscript{16}, and were maintained on a C57BL/6J background. Mice were subject to a reverse methanol/PBT series as above, washed twice with PBT for 5 min and treated with 10 μg/ml of proteinase K in PBT (E9.5 for 8 min, E10.5 for 15 min and E12.5 for 25 min). Embryos were washed in PBT twice for 5 min and post-fixed in 4% PFA with 0.2% glutaraldehyde for 40 min. Following this, embryos were washed in PBT twice for 5 min and post-fixed in 4% PFA with 0.2% glutaraldehyde for 40 min. Following this, the placenta was removed and the embryos were placed in 100% glycerol. Embryos in E12.5 for 25 min). Embryos were washed in PBT twice for 5 min and post-fixed in 4% PFA with 0.2% glutaraldehyde for 40 min. Following this, embryos were washed in PBT twice for 5 min and post-fixed in 4% PFA with 0.2% glutaraldehyde for 40 min. Following this, the placenta was removed and the embryos were placed in 100% glycerol. Embryos were fixed in 4% paraformaldehyde (PFA) and 0.1% Triton X-100 in PBS at 4 °C overnight. Embryos were then washed in PBS three times for 5 min each, gently rocking at 65 °C. Embryos were washed in PBS-T (0.1% Tween-20) three times for 5 min at RT and transferred to blocking solution (PBS-T containing 1% BSA and 0.01% Triton X-100) for 2 h at RT. Embryos were then placed into blocking solution containing 1:2000 anti-DIG antibody (Roche, 11093274910) rocking overnight at 4 °C. The next morning, embryos were washed at least five times in PBS-T for 1 h each time, and again overnight at 4 °C. The following day, embryos were washed in NTT (100 mM NaCl, 100 mM Tris-HCl (pH9.5); 0.1% Tween-20) three times for 10 min. For colour development, embryos were incubated in BM purple (Roche, 11424074001), protected from light. Colour development was stopped by washing 3 times in PBT for 5 min each and in PBS medium (composition: 0.1% Tween-20). Embryos were rinsed in PBT 3 times for 5 min each and stored in PBT at 4 °C until imaged. Plasmids for riboprobe generation were kind gifts from A. Mansouri (Uncx1.1), P. Sharpe (Hoxc6), C. Tabin (Hoxa11, Hoxd11, Hoxd12) or were generated in-house (Hoxd12, Hoxb13).

Cell lines. The Bruce4 mouse embryonic stem cell line was originally isolated from a C57BL/6 mouse\textsuperscript{16} and kindly provided by Dr Jeff Mann. Two in-house generated iPSC lines were derived from the miR-196 triple knockout mouse strain\textsuperscript{16} and wild-type mice of an isogenic background.

Generation of mouse induced pluripotent stem cells (iPSCs). iPSCs were generated from tail tip fibroblasts of adult WT and miR196a1GFP/a2Gfp/b−/− mice previously described\textsuperscript{16}, by tail tip fibroblasts were isolated and plated with standard fibroblast media containing DMEM, 10% FBS, 1x Glutamax (Gibco 35050061) and 1x Pen/Strep (Gibco 15140122). After confluence, fibroblasts were infected with a Doxycycline-inducible lentivirus expressing the OKSM cassette. Reprogramming was carried out for 16 days following the addition of Doxycycline. Colon lines were established after withdrawal from Doxycycline and maintained in ES media containing DMEM KO, 15% ES stage FBS, 1x Glutamax (Gibco 35050061), 1x Pen/Strep (Gibco 35050061), 1x NEAA and 100 μM β-mercaptoethanol and 1000 μM LIF on mitotically inactive mouse embryonic fibroblasts. Established iPSC lines were passaged every 72 h at low density.

Teratoma assay. Pluripotency of individual iPSC lines were tested by assessing their ability to form teratomas\textsuperscript{24}. Mouse iPSCs were transplanted into a feeder cell-depleted twice for 20 min. Approximately 1 × 10⁶ WT or mir196-TKO iPSCs were subcutaneously injected into the dorsal flank of isoflavone-anaesthetised immune-suppressed NOD-SCID mice of 6–8 weeks of age. Within 4–6 weeks, teratoma formation was evident and consequently removed, washed in Dulbecco’s PBS and fixed in 4% paraformaldehyde (Merck Millipore, 30252-B9) overnight at room temperature (RT). Teratomas were then embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Slides were scanned using MIAX Scan software and viewed with MIAX Viewer 1.12 software.

Determination of mouse iPSCs and ESCs. Mouse iPSCs and ESCs were differentiated according to published protocols\textsuperscript{25,26} with minor modifications. Briefly, iPSCs or ESCs were routinely maintained in ES medium (composition: 81.8% Knockout DMEM (Gibco, 10829-018); 15% foetal bovine serum; 1% Pen Strep (Gibco, 15140-122); 1% GlutaMAX-I (Gibco, 35050-061); 1% MEM NEAA (Gibco, 35050-061), 1x Pen/Strep (Gibco 35050061), 1x NEAA and 100 μM β-mercaptoethanol and 1000 μM LIF on mitotically inactive mouse embryonic fibroblasts. Established iPSC lines were passaged every 72 h at low density.
were visualised as heatmaps using ggplot2 (v3.3.2). Antibodies were added into blocking solution at the concentrations specified below: D0—10 ng/ml Bfgf (human, Miltenyi Biotec); refreshed at 24 h; D2—5 μM CHIR99021 (StemMACS, 130-103-926); D6—50 μg/ml Gdf11 (human, Miltenyi Biotec). Cells were mechanically dissociated and split at a ratio 1:3 on D3 of differentiation. For iPSC differentiation, N2B27 was supplemented as follows: D0—20 ng/ml Bfgf (human, Pепrotec); D2—10 μM CHIR99021 (StemMACS, 130-103-926); D5—50 μg/ml Gdf11 (Preprotech), with cells reseeded on D6.

**Tissue microdissection.** The entire presomitic mesoderm and adjacent tissue, caudal to the most recent formed somite, was collected from E9.5 and E10.5 embryos. Presomitic mesoderm tissue was immediately placed in RTL lysis buffer (Qiagen), frozen on dry ice and stored at −80 °C. Yolk sac tissue was collected and used for genotyping where required. The remainder of each embryo was fixed overnight in 4% paraformaldehyde at 4 °C and processed for whole-mount in situ hybridisation to detect Uncx4.1 expression and determine somite number.

**RNA extraction.** RNA from cells undergoing in vitro differentiation or cells isolated from in vivo tissue microdissection, was extracted using RNAeasy micro (QIAGEN) or Nucleospin RNA (Macherey-Nagel GmbH & Co) kits.

**Gene expression analysis by BioMark Fluidigm.** 100 ng (E9.5 and E10.5) tailbud RNA was used for qDNA synthesis performed with RT-V20 (Thermo Fisher). Quantitative PCR was performed using the 96×96 BioMark Fluidigm format. Taqman probes used are listed in Supplementary Table 5. Raw Ct values were analysed using a modified version of the qPCR-Biomark script [https://github.com/jpouch/qPCR-Biomark] and normalised as described.

**Gene expression analysis by quantitative PCR.** Additional quantitative PCR analysis was performed on a Lightcyler 480 (Roche) using the SYBR Green I Master Mix (Roche). Two microtites of cDNA was amplified per reaction using the following programme: 95 °C for 10 s (1 cycle), 95 °C for 10 s, 60 °C for 15 s, 72 °C for 10 s (45 cycles). Primers used in these studies were either previously published or synthesised in-house and listed in Supplementary Table 6.

**Dissection and tissue processing for sectioning.** Whole mouse embryos from various stages were dissected and fixed overnight in 4% PFA/PBS at 4 °C. Samples were washed twice for 10 min in PBS at 4 °C. Samples were equilibrated through a series of sucrose solutions (5%, 20%, 30% sucrose in PBS) before being embedded in OCT medium (TissueTek) using an ethanol-bath and stored at −80 °C. Frozen tissue was sectioned at 12-μm thickness on a Leica cryostome. Sections were mounted directly onto slides (Superfrost, Fisher Scientific).

**Section immunofluorescence.** Slide-mounted sections were performed as previously described. Slides were placed into blocking solution (5% heat-inactivated goat serum, 0.5% bovine serum albumin, 0.1% Triton-X in PBS) for at least 30 min. Primary antibodies were added into blocking solution at the concentrations specified below, placed onto the slides, coverslipped and incubated overnight at 4 °C in a humidified chamber. The following day, coverslips were removed and slides were washed in PBS + 0.1% Triton-X (PBSTx) 3–5 times for at least 5 min. Secondary antibodies were added to the blocking solution at 1:100, placed onto the slides, coverslipped and incubated for 1 h at RT in a humidified chamber. Slides were washed in PBSTx 3–5 times for at least 5 min. One wash included DAPI (1:1000). Slides were mounted in Prolong Diamond Mounting Media. Primary antibodies rabbit anti-T/Brachyury (T) antibody (AbCam, Ab209665) and rat anti-SOX2 antibody (AbCam, Ab92494), rabbit anti-Foxa2 (SevenHills Bioreagents, WRAB-1200) were used at 1:1000. Secondary antibodies rat-anti-AlexaFluor 488 (ThermoFisher, A21208) and anti-rabbit AlexaFluor 555 (ThermoFisher, A15752) were used at 1:1000.

Whole-mount immunofluorescence. E12.5 embryos were dissected in PBS, fixed overnight in 4% PFA at 4 °C on a rocking table. Embryos were then washed in PBS + 0.1% Tween20, dehydrated through a methanol/PBS-Tween series, and stored at −20 °C until further processing. Whole-mount immunofluorescence was performed following a published protocol, with minor modifications. Briefly, embryos were bleached in methanol + 6% H2O2 overnight at 4 °C, rehydrated through an ethanol/PBS series and stored at −20 °C at least 6 times over the course of 24 h. Primary antibodies were detected sequentially: first with a donkey anti-goat-AlexaFluor-647 (1:1000, Thermofisher, A-21414) for 3 days in PBS-GT, washed again as above, then with a goat anti-rat-AlexaFluor-555 (1:1000, Thermofisher, A-21343) and a goat anti-rabbit-AlexaFluor-790 (1:1000, Invitrogen, A11369) for another 3 days in PBS-GT. After another series of washes, embryos were cleared in 50% tetra-hydrofuran (THF)/50% H2O overnight, 80% THF/20% H2O for 2 h, 100% THF for 2 h, twice, 100% di-chloromethane (DCM) for 1 h, and 100% di-benzyl-ether (DBE). All incubations, washes, and clearing were performed on a rotating wheel at room temperature, protected from light. Cleared samples were imaged in DBE, and stored in DBE at room temperature, protected from light.

**Imaging and image processing.** Whole-mount brightfield images of E12.5 and E13.5 embryos were acquired using a NZS-405 Zoom Stereo Microscope at ×1.5 and ×4 magnifications. Images were adjusted for brightness and some images were reoriented to have the tails oriented in the same direction for easy visualisation.

Fluorescent images of slide-mounted embryonic sections were imaged with a Zeiss Axiosmat Z1 and automatically tiled on the Zeiss Imaris (Z1, Invitrogen). One image was tiled using AxioVisionRel.4.8 in-built image stitching function Mosaic. Tiled image and individual image size ratios were used to ensure direct comparisons between tiled images. Single-cell layer images of E12.5 split tail tips were acquired using Zeiss Axiosmat Z1 in Apotome mode (G2.7.5.400 mm; gfp, 423662-0000-000) and ×20 objective magnification. Linear adjustments, such as for "Brightness" or "Contrast", in which the same change is made to each pixel according to a linear function, were used on whole immunofluorescence images using Fiji (ImageJ2.0) or AxioVision Software. Fluorescence images of individual serial sections were uniformly processed using identical linear adjustments. For the cropped regions of apoptosis acquired split tail tips regions linear adjustments were made to individual images and background subtraction was applied to the single-fluorescence images to extract/distinguish the signal above autofluorescence.

Images of E18.5 skeletal preparations and E10.5 embryos following whole-mount RNA in situ hybridisations were acquired with a Vision Dynamic BK Lab System at the Monash University Paleontology Lab. Images were taken with a Canon 5d MkII with a 100 mm Macro lens (focus step 1.3/1.1). Multiple images were taken to extend the focal depth and stacked in ZereneStacker using the PMax algorithm. Scales were determined via known pixel counts for each focus stop in Adobe Photoshop.

For whole-mount immunofluorescence-stained embryos, Z sections were acquired every 2 μm on a lightsheet microscope (Ultramicroscope 2, LaVision, Miltenyi Biotec GmbH). Image stacks were imported into Imaris (v9.6; BitPlane, Oxford Instrument) and visualised in 3D. Contrast was adjusted for each channel and segmentation was performed using the Surface tool. Pictures and animations were created in Imaris.

Adobe Illustrator was used to compose all multipanel figures in this study.

**Statistics and reproducibility.** All skeletal phenotyping was performed independently by two investigators, blinded to genotype. No statistical method was used to predetermined sample size. For gene expression analyses, Ct values greater than the manufacturer recommended threshold were excluded.

**Data availability.** The raw Fluidigm qPCR data generated in this study are provided in the Source data file. Summary tables of skeletal scoring data are available as supplementary tables 1–3. Supplementary Movies are available under the link: [https://figshare.com/s/78383c7beb9b85c959bf](https://figshare.com/s/78383c7beb9b85c959bf). Source data are provided with this paper.

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**References.**

1. Bénazéraf, B. & Pourquié, O. Formation and segmentation of the vertebrate body axis. *Annu. Rev. Cell Dev. Biol.* 29, 1–26 (2013).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
2. Wellik, D. M. Hox patterning of the vertebrate axial skeleton. Dev. Dyn. 236, 2454–2463 (2007).
3. Cunha-Costa, M., Novoa, A., Bobola, N. & Mallo, M. Hox genes specify vertebral types in the presomatic mesoderm. Genes Dev. 19, 2116–2121 (2005).
4. Bateson, W. Materials for the Study of Variation Treated with Special Regard to Discontinuity in the Origin of Species. https://doi.org/10.5962/bhl.title17250 (1894).
5. Crompton, R. Descriptive Catalogue of the Osteological Series Contained in the Museum of the Royal College of Surgeons of England (Royal College of Surgeons, 1853).
6. Narita, Y. & Kuratani, S. Evolution of the vertebral formula in mammals: a developmental origin of aberrant cervical anatomy in tree sloths. Evol. Dev. 11, 69–79 (2009).
7. Galis, F. & Metz, J. A. J. Evolutionary novelties: the making and breaking of pleiotropic constraints. Integr. Comp. Biol. 47, 409–419 (2007).
8. Sánchez-Villagra, M. R., Goswami, A. & Asher, R. J. Skeletal development in sloths and the evolution of mammalian vertebral patterning. Proc. Natl Acad. Sci. USA 107, 18963–18968 (2010).
9. Robinton, D. A. et al. The Lin28/let-7 pathway regulates the mammalian growth in mammalian embryos. Cell 67, 89–104 (1991).
10. Pollock, R. A., Jay, G. & Bierberch, C. J. Altering the boundaries of Hox3.1 expression: evidence for antipodal gene regulation. Cell 71, 911–923 (1992).
11. Wellik, D. M. & Capocci, M. R. Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. Science 303, 363–367 (2003).
12. Ye, Z. & Kimelman, D. hox13 genes are required for mesoderm formation and axis elongation during early zebrafish development. Development 185298, https://doi.org/10.1242/dev.185298 (2020).
13. Benahmed, F. et al. Multiple regulatory regions control the complex expression pattern of the mouse Cdx2 homeobox gene. Gastroenterology 135, 1238–1247, 1247.e1–3 (2008–2009).
14. Denans, N., Imura, T. & Pourquié, O. Hox genes control vertebral body elongation by collinear Wnt repression. Elife 4, e04379 (2015).
15. Economides, K. D., Zeltser, L. & Capecchi, M. R. Hoxb13 mutations cause overgrowth of caudal spinal cord and tail vertebrae. Dev. Biol. 256, 317–330 (2003).
16. Runck, L. A. et al. Defining the molecular pathways in cloaca malformation: similarities between mouse and human. Dev. Model. Mech. 7, 483–493 (2014).
17. Orton, G. et al. Hox13 gene expression in the caudal metanephros of the mouse. Development 146, 168161 (2019).
18. Fellon, J. F. & Simandl, B. K. Evidence of a role for cell death in the disappearance of the embryonic human tail. Am. J. Anat. 152, 111–129 (1978).
19. Sawin, P. B. Morphogenetic studies of the rabbit; regional specificity of hereditary factors affecting homeoctic variations in the axial skeleton. J. Exp. Zool. 100, 301–329 (1945).
20. Deschamps, J. & Duboule, D. Embryonic timing, axial stem cells, chromatin dynamics, and the Hox code. Dev. Dyn. 231, 146–1416 (2017).
21. Forlani, S., Lawson, K. A. & Deschamps, J. Acquisition of Hox codes during axial progenitors controls motor neuron subtype specification. Development 149, e194514 (2021).
22. Kessel, M. & Gruss, P. Homeostatic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. Cell 67, 89–104 (1991).
23..Runck, L. A. et al. De...
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Author contributions

Conceptualisation, methodology and formal analysis, G.M.H., V.C.G. and E.M.; investigation, G.M.H., V.C.G., S.F.L.W., H.B., J.M., Y.C., C.M.N., J.F., J.C., F.J.R. and E.M.; writing—original draft, G.M.H. and E.M.; writing—review and editing, all authors; funding acquisition, E.M. and J.M.P.; supervision, F.J.R., J.M.P. and E.M.

Competing interests

The authors declare no competing interests.

Additional information

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