A Novel Gain-Of-Function Mutation of the Proneural IRX1 and IRX2 Genes Disrupts Axis Elongation in the Araucana Rumpless Chicken

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Abstract

Axis elongation of the vertebrate embryo involves the generation of cell lineages from posterior progenitor populations. We investigated the molecular mechanism governing axis elongation in vertebrates using the Araucana rumpless chicken. Araucana embryos exhibit a defect in axis elongation, failing to form the terminal somites and concomitant free caudal vertebrae, pygostyle, and associated tissues of the tail. Through whole genome sequencing of six Araucana we have identified a critical 130 kb region, containing two candidate causative SNPs. Both SNPs are proximal to the IRX1 and IRX2 genes, which are required for neural specification. We show that IRX1 and IRX2 are both misexpressed within the bipotential chordoneural hinge progenitor population of Araucana embryos. Expression analysis of BRA and TBX6, required for specification of mesoderm, shows that both are downregulated, whereas SOX2, required for neural patterning, is expressed in ectopic epithelial tissue. Finally, we show downregulation of genes required for the protection and maintenance of the tailbud progenitor population from the effects of retinoic acid. Our results support a model where the disruption in balance of mesoderm and neural fate results in early depletion of the progenitor population as excess neural tissue forms at the expense of mesoderm, leading to too few mesoderm cells to form the terminal somites. Together this cascade of events leads to axis truncation.

Introduction

During secondary body formation the regressing primitive streak and Hensen’s node are transformed into a bulblike structure, the tailbud, a morphologically uniform mass of mesenchyme [1,2,3]. The tailbud mesenchyme is located adjacent to the posterior end of the neural tube and notochord, an area known as the chordoneural hinge (CNH) [4,5]. The CNH together with the dorso-posterior and ventral tailbud populations gives rise to all the derivatives of the tail [6,7]. These include the precursor to the secondary neural tube (the medullary cord), somite progenitors of the presomitic mesoderm, and the posterior extension of the notochord [3,4,6]. The CNH acts as a bipotential population of long-term axial progenitors, contributing cells to both the somitic mesoderm and medullary cord neuroectoderm [6,7,8]. The bipotential nature of the CNH requires fine control of expression of neural genes such as SOX2 and mesodermal genes such as TBX6 in order to maintain the balance of neural and mesodermal cell fates [9,10,11].

Throughout secondary body formation the remaining somites form from the paraxial mesoderm (PM) of the tail [12]. Somites are transitory paired epithelial spheres that differentiate to generate the axial skeleton, including the vertebrae, cartilage, and most of the skeletal musculature and dermis [13]. The number of somites formed is species-specific and highly variable; for example whereas chicken (Gallus gallus) has between 51–53 somites, mouse (Mus musculus) has approximately 65 somites and the corn snake (Pantherophis guttatus) over 300 somites [14,15]. Somitogenesis is a critical process during axis elongation, and interference brought about by teratogenic factors or the existence of congenital mutations can lead to axis truncation. Many model organisms have been used to study axis elongation including chicken, mouse, and zebrafish [7]. Notably in mice, spontaneous mutants such as the vestigial tail mouse, hypomorph for Wnt3a, and the Brachyury (T) mouse have been studied as models of axis truncation [16,17,18,19]. Null or reduced expression of either Wnt3a or T leads to a failure to maintain the CNH progenitor population and failure of mesoderm specification. Additionally, gene knockouts of FGF8 and CYP26A1 also result in axis truncation, with the latter required to protect the progenitor population from the apoptotic effects of retinoic acid until extension is complete [20,21]. Current models of axis length...
termination include the elimination of the tailbud progenitor population through programmed cell death and diminution of the presomitic mesoderm (PSM) [22,23,24,25]. However, many gaps in knowledge still exist, requiring a better understanding of the genes, signals and regulators of secondary body formation and its subsequent termination.

The Araucana chicken breed has been maintained as show birds for their rumpless (\(Rp\)) and ear tuft morphology [26,27,28,29,30]. Rumplessness is an inherited autosomal dominant disorder, which we had previously mapped to a 740 kb region on chromosome 2 [28,31]. The Araucana model offers an opportunity to further elucidate the morphogenetic and molecular mechanisms required for normal tail development, as well as the cessation of axis elongation, in an accessible model organism. Here, we investigate the mechanism of rumplessness and the identity of the causative mutation.

In the current study we show that misexpression of the IRX1 and IRX2 proneural genes, located within our candidate region, precedes a cascade of altered downstream gene expression. This results in a morphogenetic chain reaction including: changes in bipotential progenitor cell fate, premature depletion of progenitors, early termination of somitogenesis, and early apoptosis of the progenitor remnant and posterior axis malformation. Furthermore, we identify two candidate causative mutations, within a narrowed 130 kb region of chromosome 2 through bioinformatics analysis of whole genome resequencing of six Araucana birds. Together, our results provide a greater understanding of the mechanism of secondary body formation, cell fate determination, axial elongation, determination of posterior somite numbers and control of overall tail length.

Materials and Methods

Animals

Clemson University IACUC approved the study, protocol number 2011-041. Fertilized chicken eggs were obtained from SkyBlueEgg (Arkansas, U.S.A.) and the Clemson University Poultry Farm. Eggs were incubated at 38.5°C in a humidified chamber to the desired stage. Embryos were staged according to Hamburger and Hamilton (HH) [32]. Skeletal material was the gift of the Araucana Club of America.

Bone and cartilage staining

Bone and cartilage staining was carried out on E18 Araucana\(^{Rp}\) and tailed controls using Alcian blue (Polysciences) and Alizarin red S (Acros Organics) according to standard procedures [33]. Briefly, Embryos were fixed 3 \times 24 hours in 95% EtOH, 100% EtOH, 2 \times 24 h in 100% Acetone. Cartilage staining (20 mg Alcian Blue in 100 ml of 40% acetic acid glacial/EtOH) was performed from a few hours to overnight depending on sample size. Embryos were rinsed in EtOH for 15 min followed by EtOH for 24 hrs. They were then placed in saturated borax solution 2 \times 24 hours (\(\text{Na}_2\text{B}_4\text{O}_7\cdot 10\text{H}_2\text{O}\) in H\(_2\)O). Trypsin solution (0.45 g purified trypsin in 400 mL of 30% borax dissolved in distilled water) at 30°C was used to clear tissue until flesh became translucent and soft (between 1-4 days, depending on size of sample). Alizarin Red S solution (0.5% KOH and 0.1% Alizarin Red S) was used to stain bones (12-24 hours). Samples were then washed in distilled water, followed by a wash in 0.5% KOH solution for 15 min. Excess Alizarin Red S stain was removed using 0.5% KOH solution for 2 \times 24 hours at room temperature under a light source. Samples then went through series of glycerol 0.5% KOH washes (20% glycerol/0.5% KOH, 50/50 and 75/25 mix). Samples were stored in 100% glycerol with 100 mg thymol crystals.

Somite number counts

Araucana\(^{Rp}\) and controls were incubated to between HH16-25. Embryos were harvested and somite counts performed using a Nikon stereoscopic microscope (control \(n = 73\), Araucana\(^{Rp}\) \(n = 83\)). At later stages, between HH22-25, DACT2 ISH labeling was used to aid counts of the posterior somites. The number of somites in tailed controls was compared against the expected number of somites as described in the normal stage series, and found to match [32].

Statistical Analysis

Assuming a normal distribution of the data, a two-tailed \(t\)-test was carried out to test for differences in the average values of samples from experiments for somite counts, proliferation, and TUNEL. Analysis was carried out using Statistical Analysis Software (SAS).

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA/PBS) for 48 hours before being cryoembedded in 15% sucrose/7.5% gelatin/PBS and sectioned on a Leica cryotome at 25 \(\mu\)m. Immunostaining was carried out using our standard protocol [34]. Briefly, sections were blocked in PBS with 0.1% Triton-X-100 and 0.2% bovine serum albumin (BSA). Then incubated overnight at 4°C with the primary antibodies anti-E-cadherin (cat 610182, BD Bioscience) and anti-laminin (cat L9393, Sigma). Following washing in PBS sections were incubated at secondary antibodies 1:200 Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen). Following washing in PBS and mounting with SlowFade (Life Technologies), fluorescent images were captured using a Nikon Ti Eclipse confocal microscope.

In situ hybridization

Whole-mount in situ hybridization (ISH) was performed according to our standard procedures using probes against \(BRA\) (control \(n = 18\), Araucana\(^{Rp}\) \(n = 14\)), \(CYP26A1\) (control \(n = 16\), Araucana\(^{Rp}\) \(n = 8\)), \(DACT2\) (control \(n = 19\), Araucana\(^{Rp}\) \(n = 11\)), \(FGF8\) (control \(n = 19\), Araucana\(^{Rp}\) \(n = 11\)), \(IRX1\) (control \(n = 26\), Araucana\(^{Rp}\) \(n = 12\)), \(IRX2\) (control \(n = 25\), Araucana\(^{Rp}\) \(n = 17\)), \(IRX4\) (control \(n = 16\), Araucana\(^{Rp}\) \(n = 9\)), \(MESO1\) (control \(n = 17\), Araucana\(^{Rp}\) \(n = 9\)), \(RALDH2\) (control \(n = 8\), Araucana\(^{Rp}\) \(n = 8\)), \(SOX2\) (control \(n = 6\), Araucana\(^{Rp}\) \(n = 9\)), \(TBX6\) (control \(n = 29\), Araucana\(^{Rp}\) \(n = 19\)), and \(WNT3A\) (control \(n = 18\), Araucana\(^{Rp}\) \(n = 13\)) [34]. Probes have all been previously described as follows: \(DACT2\), \(MESO1\) and \(TBX6\) [24], \(FGF8\) [35], \(IRX1\), \(IRX2\) and \(IRX4\) [36], \(RALDH2\) [37] and \(WNT3A\) [38]. \(IRX1\), \(IRX2\), and \(IRX4\) probes were the generous gift of Dr. Cherylly Tickle. Embryos were cryoembedded in 15% sucrose, 7.5% gelatin/PBS and sectioned on a Leica cryotome. Whole mount embryos and sections were imaged on a Nikon Smz1500 stereomicroscope and Nikon Eclipse 80i compound microscope, respectively using a Qimaging Micropublisher 5.0 camera.

EdU and TUNEL labeling

For proliferation analysis, Click-iT EdU 488 Imaging Kit (Invitrogen) was used to carry out labeling of cells as previously described [39]. Briefly, embryos were pulsed with EdU for 60 minutes before harvesting and fixation in 4% PFA overnight. Embryos were then cryoembedded in 15% sucrose/7.5% gelatin/PBS and sectioned on a Leica cryotome at 25 \(\mu\)m. Alternating
sections were processed for EdU detection with a counterstain of TO-PRO-3 iodide, or apoptosis detection using the TUNEL method (In Situ Cell Death Detection Kit, TMR red, Roche) with a Hoechst counterstain, and imaged on a Nikon Ti Eclipse confocal microscope. For each sample, a single matching EdU and TUNEL labeled medio-lateral image was selected. Image analysis of EdU, TUNEL, and TO-PRO-3 labeling was carried out using ImageJ (control n = 21, Araucana<sup>Rp</sup> n = 17). For individual cell counts of EdU, TO-PRO-3, and TUNEL stained cells, a region of interest was manually selected consisting of the tailbud mesenchyme based on location and morphology, excluding the surrounding ectoderm.

Whole genome sequencing and bioinformatics
DNA samples for six Araucana were acquired from our previous study [31]. Each of the six samples was sequenced on six lanes with an Illumina HiSeq 2000 sequencer. Average genomic coverage was 27.63x and average number of bases sequenced was 29,009 Giga base pairs (Table S1). Sequence reads were trimmed using Trimmomatic and aligned to the corresponding region previously identified to be associated with the rumpless phenotype on chromosome 2 of the ICGSC Gallus_gallus-4.0/gaGal4 build using Bowtie2 applications [40,41]. The mpileup function of SamTools was used to call variants [42]. The view option of bcftools was used to call the genotype at each variant for each individual bird using the bcftools defaults. Variants that were found to be homozygous in all three homozygous rumpless birds, found to be heterozygous in all two heterozygous birds, and were not found in the homozygous tailless bird were considered fixed in each individual bird using the bcftools defaults. Variants that were previously identified not to be involved in rumplessness were removed [43].

Results

Araucana<sup>Rp</sup> lack the caudal-most vertebrae
The current North American breed standard of the Araucana chicken requires that they lack the caudal vertebrate and other tail structures, appearing rumpless (Fig. 1A). This distinctive morphology is revealed by comparison of adult tailless control and rumpless Araucana (Araucana<sup>Rp</sup>) skeletons, where Araucana<sup>Rp</sup> lack the free caudal vertebrae and pygostyle of the tail (Fig. 1B-C). By performing cartilage and bone staining (Alician Blue and Alizarin Red S) from embryonic day E5 to E18, we determined that failure to form the vertebrae, rather than reabsorption occurs. At E18, 5 free caudal vertebrae and 6 fused vertebrae of the pygostyle are observed in controls (Fig. 1D), but are missing in Araucana<sup>Rp</sup> (Fig. 1E). From these data we conclude that the rumpless phenotype observed in the Araucana<sup>Rp</sup> adult chicken arises during early posterior development due to the lack of free caudal vertebrae and the pygostyle, matching previous observations of rumpless chickens [20].

Araucana<sup>Rp</sup> embryos display truncated tail morphology at the tail organizer stage
To examine potential mechanisms resulting in axis truncation, we first determined the earliest stage at which Araucana<sup>Rp</sup> embryos displayed morphological differences by examining the gross morphology of the tail region. Formation of the tailbud occurs at HH14-15 and is identical between controls and Araucana<sup>Rp</sup> [1,2,3]. At HH16, the Araucana<sup>Rp</sup> embryo tail appeared less elongated and more pointed than rounded compared to controls (Fig. 2A-B). Moreover, a lesser angle of curvature between the Ventral Ectodermal Ridge (VER) and extraembryonic ectoderm was apparent (arrow, Fig. 2A-B). This became more pronounced at HH18, with reduced distance between the most recently formed somite pair (asterisk) and the end of the tail in Araucana<sup>Rp</sup> (Fig. 2C-D). The tip of the tail appeared pointed and the mesenchymal cells underlying the ectoderm cap had dense apoptotic morphology. By HH20 elongation of the tail has ceased in Araucana<sup>Rp</sup>, with the most recently formed somite located more posteriorly in the tail than controls (asterisk, Fig. 2E-F).

Araucana<sup>Rp</sup> embryos downregulate TBX6 and BRA at the tail organizer stage
Having identified the critical stage at which morphology was affected we next analyzed TBX6 and BRA expression. Both T-box transcription factors are important for proper axial elongation, with mutations leading to changes in either gene’s expression causing axis truncation [10,11,17,18,19,44,45,46]. TBX6 is a marker of the presomitic mesoderm (PSM) and undifferentiated mesenchyme in the tailbud (asterisk, Fig. 2G). Starting to indirectly repress the neural transcription factor SOX2 in order to specify mesoderm (Fig. 2G,I,K) [9,11]. Up to HH15 TBX6 expression was as expected in control and Araucana<sup>Rp</sup> embryos. At HH15 (26 pairs of somites), TBX6 expression was downregulated in the undifferentiated mesenchyme of the Araucana<sup>Rp</sup> tailbud (asterisk, Fig. 2H, inset). The tailbud comprises all mesenchyme posterior to the neural tube and notochord, consisting of the chordoneural hinge population (CNH) and tailbud mesenchyme (TBM) that lies directly underneath the ectoderm capping the tail [3,6]. Expression of TBX6 was maintained in the PSM in HH15-HH17 embryos, but not the undifferentiated mesenchyme of the TBM (Fig. 2Hj). However, by HH20 TBX6 expression within the PSM of Araucana<sup>Rp</sup> embryos was no longer visible (Fig. 2K,L). This suggests that following downregulation of TBX6 within the tailbud, Araucana<sup>Rp</sup> fail to specify new PSM.

BRA is a marker of the TBM and notochord, and is required for mesoderm specification in the tail (Fig. 2M,O,Q) [17,18,44]. Heterozygous T mutant mice display malformed sacral vertebrae and shortening of the tail due to a failure of axis elongation and somite formation during embryogenesis [18,19,44]. Loss of BRA expression in Araucana<sup>Rp</sup> followed downregulation of TBX6 within the tailbud mesenchyme at HH16, whereas notochord expression of BRA remained unaffected (Fig. 2N,P,R). These results show that the defect leading to Araucana<sup>Rp</sup> rumplessness arises early in tailbud development, and involves the downregulation of transcription factors required for specification of the PSM. Observation of the downregulation of TBX6 and BRA within the TBM, which contributes to the PSM, suggests a failure of new PSM specification in Araucana<sup>Rp</sup> embryo tails as early as HH15 (26 somites) [6].

Somitogenesis ends prematurely in Araucana<sup>Rp</sup> embryos
In tailless embryo axial elongation and somitogenesis continue until HH24-25, when 51–53 somite pairs have formed [24]. Due to the downregulation of TBX6 and BRA and their requirement to specify PSM, we predicted Araucana<sup>Rp</sup> would fail to form the correct number of somite pairs [11,44]. To that end we analyzed MESO1 expression. MESO1 is the homolog of mouse Mesp2, a transcription factor expressed in the anterior presomitic mesoderm that plays a role in somite segment border formation, and is required for the formation of the next pair of epithelial somites (Fig. 3A,C) [47,48]. TBX6 binds to regulatory elements of Mesp2 and is required for its expression [49].
expression is normally downregulated at HH24-25, marking the end of somitogenesis [24]. We found that MESO1 expression matched controls (Fig. 3A–B) until HH19, at which point MESO1 expression was lost in AraucanaRp (Fig. 3C–D). MESO1 expression was lost as TBX6 was downregulated within the remaining PSM in AraucanaRp (Fig. 2L and 3D). This result suggests that AraucanaRp somite formation is arrested as early as HH19.

Next we examined expression of the homolog of zebrafish dpr2, DACT2, a regulator of WNT and TGFβ signaling that is expressed in the anterior primitive streak, neural crest cells, and most recently formed somites after HH17 [24,50,51,52]. DACT2 expression in AraucanaRp labeled a similar number of recently formed somites when compared to controls at HH19 (Fig. 3E–F). However, the distance from the most posterior somite to the tip of the tail was shortened, suggesting less PSM in AraucanaRp than controls (Fig. 3G,H). This suggests that somite formation is reduced in HH20 AraucanaRp as fewer DACT2 labeled somites equates to a lack of further somite formation. In controls, DACT2 continued to label recently formed somites through the end of somitogenesis at HH24-25 [24,52]. The downregulation of MESO1 and DACT2 expression 22.5 hours earlier than in controls indicates that the most posterior somites, which give rise to the free caudal vertebrae and pygostyle, fail to form.

To confirm the lack of somite formation we performed somite counts for AraucanaRp and controls from HH16-25 (Fig. 3I).

Figure 1. Skeletal analysis shows AraucanaRp lack caudal vertebrae. Adult AraucanaRp male and female birds shown in a composite image (A) (courtesy of Fritz Ludwig). Note the characteristic rounded rump, lacking tail structures. Skeletons of control (B) and AraucanaRp (C) birds (courtesy of the ACA). The free vertebrae and pygostyle are missing in the AraucanaRp skeleton (arrow). E18 embryos stained with Alcian Blue in control (D) and AraucanaRp (E). AraucanaRp embryos lack the free vertebrae and pygostyle. Arrowheads indicate lateral processes. Vertebral elements are numbered from the first free vertebrae (1–5). The more posterior vertebral elements (6–11) fuse to form the mature pygostyle after hatching. FV-free caudal vertebrae, P-pygostyle, S-sacral vertebrae.

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Figure 2. AraucanaRp embryo tailbud is truncated and downregulates TBX6 and BRA. Whole mount tails in lateral view, with posterior at the bottom, dorsal to the left (A–F). At HH16, the angle of tail curvature is narrower in controls (A) compared to AraucanaRp embryos (B-arrowhead). By HH18, the reduced length and pointed shape of the AraucanaRp tail is dramatic (D) compared to controls (C). The control tail has curved ventrally by HH20 (E), whereas the AraucanaRp tail has failed to extend (F). Asterisks denote level of posterior-most somite pair. Somite formation in AraucanaRp is near the tip of the tail (F-asterisk). Expression patterns of TBX6 (G-L) and BRA (M–R) during tailbud development. ISH expression of TBX6 in control (G,K) and AraucanaRp (H,J,L) at HH15, 17 and 20. Inset in G and H are transverse sections of respective embryos at level of tailbud, asterisk denotes undifferentiated mesenchyme. Note downregulation of TBX6 expression in AraucanaRp (arrowheads) compared to controls. ISH expression of BRA in control (M,O,Q) and AraucanaRp (N,P,R) at HH16, 17, and 20. Inset in M and N are transverse sections of respective embryos at level of tailbud. Note loss of BRA expression in tailbud mesenchyme in AraucanaRp (arrowheads) versus controls.

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Beginning at HH20, the number of somites in AraucanaRp embryos was significantly different than in controls (p<0.01, Fig. 3I). At HH20 AraucanaRp embryos averaged 4 fewer somites than controls, and by the end of somitogenesis at HH25 AraucanaRp embryos had on average 11 fewer somites than controls. These data are consistent with our bone and cartilage labeling data in which embryos lacked the 5 free caudal vertebrae compared to control embryos. The main question that arises from these observations is what is the functional mechanism leading to axial truncation? Several possibilities present themselves, including: changes in proliferation and apoptosis in the CNH population, changes in the balance of cell fates within the bi-potential progenitor population, and changing molecular signaling within the tissues [9,22,23,24,25]. While none of these is mutually exclusive it is logical to assume that there is a single molecular event that triggers a cascade of downstream changes that eventually results in axial truncation. We next examine each of these possibilities to determine the sequence of events taking place in affected AraucanaRp embryos.

**IRX1 and IRX2 are misexpressed in the AraucanaRp embryo tailbud**

We previously identified a critical region associated with rumplessness in Araucana, containing two genes, *iroquois 1 (IRX1)* and *iroquois 2 (IRX2)* [31]. Iroquois-class homeodomain proteins play multiple roles during pattern formation, with one of their primary roles being the initial specification of the vertebrate neuroectoderm [53,54]. Importantly, *IRX1* and *IRX2* expression is normally excluded from the tailbud. To investigate expression of *IRX1* and *IRX2* in AraucanaRp embryos we performed in situ hybridization from HH10-HH20.

Expression of *IRX1* in control embryos is restricted to the neural tube of the elongating posterior axis (Fig. 4A,C,E). Analysis of *IRX1* expression up to HH14 revealed identical expression between controls and AraucanaRp (Fig. 4A-B). At HH15, *IRX1* expression in the neural tube remains unaffected in AraucanaRp (Fig. 4C–D,M), whereas *IRX1* is misexpressed medially at the level of the CNH progenitor population (Fig. 4C–D,N–P). *IRX1* misexpression occurred one somite pair earlier than downregulation of *TBX6* expression (Fig. 2H,4D). *IRX1* misexpression in AraucanaRp tailbud continued at HH16 (Fig. 4E–F) and HH17 (not shown), but by HH18 normal expression was restored (data not shown). These results illustrate a critical time window of *IRX1* misexpression at the onset of tailbud formation and the tail organizer stage.

We then analyzed the expression of *IRX2*, which is also expressed in the neural tube, but restricted more anteriorly than *IRX1* (Fig. 4G,1,K). Analysis of *IRX2* at HH14 revealed no difference in expression between controls and AraucanaRp (Fig. 4G–H). However, *IRX2* was misexpressed in AraucanaRp embryos at HH15 (Fig. 4I–J). The subpopulation of cells appeared to overlap that of *IRX1* to a limited degree, with a more ventro-lateral subpopulation of mesenchyme labeled (Fig. 4Q). *IRX2* misexpression continues at HH16, but is no longer observed from HH17 (Fig. 4K–L and data not shown).

Although *IRX4* does not fall within the defined critical region, it is within the same genomic cluster as *IRX1* and *IRX2* [31,55]. *IRX4* expression is restricted primarily to the heart at these stages, suggesting that the regulatory elements controlling its expression are not as closely shared as for *IRX1* and *IRX2* [55,56,57,58].ISH analysis from HH14-16 revealed identical expression between control and AraucanaRp embryos (data not shown).

In summary, both *IRX1* and *IRX2* are misexpressed within the AraucanaRp tailbud at the level of the CNH beginning at HH15. This aberrant expression raises two important questions. First, what is the causative mutation resulting in the gain-of-function of two proneural genes within the mesenchyme progenitor population, which commits cells to both mesodermal and neuroectodermal lineages?

**Sequencing of the critical region reveals candidate mutations**

We previously identified a 740 kb critical region associated with the rumplessness containing both *IRX1* and *IRX2* [31]. In order to identify candidate causative mutations within this region we
performed whole genome sequencing on DNA from six Araucana birds. After aligning reads to chicken chromosome 2 (GGA2), small variants (insertions, deletions, and SNPs) were called using the mpileup function of SamTools [42]. Variants were separated into three haplotype groups: three homozygous rumpless, two heterozygous rumpless, and one homozygous tailed Araucana. A total of 2092 unique small variants were identified within the candidate region when compared to the chicken Galgal4 reference sequence. We reduced the list of variants by excluding those that did not hold true to type; keeping only those that occurred in both

Figure 4. IRX1 and IRX2 are misexpressed in AraucanaRp tailbud. IRX1 expression pattern (A–F,M–P). (A,C,E) Control embryos express IRX1 in the neural tube. IRX1 expression in controls matches AraucanaRp at HH14 (A,B), but is misexpressed in AraucanaRp tailbud at HH15 (D-arrowhead). Normal expression at level of somites is within neural tube (transverse section, M). Misexpression in AraucanaRp can be seen at the level of the chordoneural hinge (transverse section-N and sagittal section-P) compared to expression in HH15 control (sagittal section-O). Misexpression is maintained through HH16 in AraucanaRp (F-arrowhead) as compared to control (E). IRX2 expression pattern (G–L,Q). (G,I,K) Control embryos do not express IRX2 in the tailbud. No difference in expression between controls and AraucanaRp seen at HH14 (G,H). IRX2 is misexpressed in HH15 (J-arrowhead) and HH16 (L-arrowhead) AraucanaRp. Transverse section of IRX2 (Q) shows expression similar to IRX1 at level of chordoneural hinge. A–L - anterior to top. M,N,Q - dorsal to top. O,P - dorsal to left, anterior to top. Abbreviations: nt-neural tube, s-somite, n-notochord.

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alleles in the homozygote, a single allele in the heterozygotes, and not present in the tailed Araucana. A total of 316 small variants matched this pattern within the critical region. A further 18 variants that lined up with previously reported variants in tailed birds were also removed [43]. Of the remaining 298 small variants, we identified 274 SNPs and 24 insertion/deletions unique to rumpless Araucana (Fig. 5 and Table S2). None of the identified 298 small variants fell within the sequenced exons or introns of IRX1 or IRX2, suggesting that the causative mutation is within the surrounding regulatory region.

During analysis of the small variants, a promising 55 base pair deletion on chromosome 2 between base pairs 86,830,975–86,831,090 was found to segregate with rumplessness. PCR analysis of the deletion was carried out on 131 Araucana\(^{bp}\), 30 tailed Araucana, and 38 non-Araucana tailed birds. In 190 cases, the deletion segregated with the phenotype correctly. However, in one case the genotype of a tailed Araucana male (RB1) did not segregate correctly. Six SNPs throughout the previously identified critical region and three newly identified SNPs from the whole genome sequencing were analyzed to identify the haplotype of RB1 (Fig. 5). RB1 was found to share only part of the rumpless haplotype, delimiting a new critical interval of 125,146 base pairs within the established Araucana haplotype block. Rumpless Araucana\(^{bp}\) haplotype from tailed Araucana in yellow. New critical interval defined by RB1 tailed Araucana highlighted by dashed lines.

Expansion of neural tissue within Araucana\(^{bp}\) embryo tailbud

Ir\(quo\)ais genes are involved in specifying and patterning neural domains [53,54]. In Xenopus, over-expression of ir\(quo\)ais causes the neural plate to expand, and promotes the onset of neural differentiation [59,60]. Importantly, cells of the tailbud have a bipotential fate, becoming mesoderm (paraxial mesoderm) or neuroectoderm (neural tube) [6,8,9,61]. Considering the misexpression of the proneural genes IRX1 and IRX2 and the premature downregulation of PSM marker TBX6, we predicted that the remaining unspecified progenitor cells would be pushed towards a neural fate. As TBX6 plays an indirect role in repressing the enhancer of the neural marker SOX2 we tested the expression of SOX2 in Araucana\(^{bp}\) embryos [9,62]. We found that beginning at HH18, Araucana\(^{bp}\) embryos displayed ectopic SOX2 expression, labeling an expanded domain of neural tissue (Fig. 6A–D). In addition, the Araucana\(^{bp}\) neural tube displayed expanded epithelial structures with multiple open and irregular lumens (Fig. 6E–F). WNT3A is expressed in the dorsal midline of the neural tube. At HH22, expression of WNT3A in control embryos was limited to the dorsal neural tube, whereas in Araucana\(^{bp}\) embryos WNT3A expression was dramatically altered within the expanded neuroepithelium of the tailbud region (Fig. 6G–H).

Thus, our results demonstrate aberrant neural differentiation in the Araucana\(^{bp}\) tail as demonstrated by ectopic neural tissue labeled with both SOX2 and WNT3A. This ectopic expression suggests an increase in the number of cells specified to a neural fate. This opens up the question of whether the shift in fate is at the expense of the mesoderm, and therefore the cause of the reduction in PSM in Araucana\(^{bp}\).

Gene expression in the retinoic acid pathway is altered in Araucana\(^{bp}\) embryos

As the tail elongates and tail mesenchyme cells are displaced anteriorly they are exposed to an increasing concentration of retinoic acid (RA) from the somites [13,25]. This exposure to RA induces differentiation, as well as plays a role in defining somite segment boundaries at early stages [63,64]. However, ectopic exposure of the tailbud to RA leads to downregulation of WNT3A and FGF8, neural expansion, and a decrease in the PSM [24,25,65]. Therefore, we evaluated the expression of RALDH2,
Acytose expression of 

\[ \text{ISH expression in control (G) and Araucana} \]

\[ \text{as HH16, CYP26A1 is downregulated in the Araucana} \]

\[ \text{progenitor mesenchyme (Fig. 7C–D). Thus, although no differences in} \]

\[ \text{RALDH2 expression were observed, lack of CYP26A1 in} \]

\[ \text{Araucana} \]

\[ \text{could lead to increased levels of RA within the} \]

\[ \text{progenitor mesenchyme. However, if levels of RA in the tail were} \]

\[ \text{higher in Araucana} \]

\[ \text{we would expect to see a downregulation of} \]

\[ \text{WNT3A and FGF8 within the tailbud [16,24,25].} \]

\[ \text{WNT3A is expressed within the tailbud where it is necessary} \]

\[ \text{to specify somite from the bipotential population, and is involved} \]

\[ \text{in the proliferation of mesoderm within the tailbud} \]

\[ [16,67,68,69,70]. \]

\[ \text{WNT3A also regulates expression of} \]

\[ \text{FGF8 within the tailbud, where FGF8 acts to maintain expression of} \]

\[ \text{CYP26A1 as well as inhibit expression of} \]

\[ \text{RALDH2, thereby creating an opposing gradient to RA} \]

\[ [64,71,72,73]. \]

\[ \text{This can be disrupted through ectopic exposure to RA in the tailbud, which} \]

\[ \text{leads to the downregulation of both} \]

\[ \text{WNT3A and FGF8, and} \]

\[ \text{cessation of further axial elongation [24,25,65,74,75].} \]

\[ \text{We found that until HH16, WNT3A expression within the} \]

\[ \text{tailbud was indistinguishable between control and Araucana} \]

\[ \text{embryos, but at HH17 (Fig. 7E,F) WNT3A expression became} \]

\[ \text{significantly downregulated in Araucana} \]

\[ \text{embryos and was lost by HH18 (Fig. 7G,H). We next examined the expression of} \]

\[ \text{FGF8 within the tailbud, where it is expressed throughout tail elongation} \]

\[ (\text{Fig. 7I,K). In Araucana} \]

\[ \text{expression was downregulated as early as} \]

\[ \text{HH17 (Fig. 7F), and by HH18 FGF8 transcripts were} \]

\[ \text{undetectable (Fig. 7L).} \]

\[ \text{These data suggest that although there is no change in} \]

\[ \text{expression of} \]

\[ \text{RALDH2, the downregulation of the RA degrading} \]

\[ \text{enzyme CYP26A1 would allow for the exposure of the tailbud to} \]

\[ \text{RA. Exposure of the tailbud to RA following the downregulation of} \]

\[ \text{CYP26A1 would explain the subsequent downregulation of} \]

\[ \text{WNT3A and FGF8.} \]

\[ \text{Araucana} \]

\[ \text{tails have reduced proliferation and} \]

\[ \text{increased apoptosis} \]

\[ \text{The premature downregulation of mesodermal markers} \]

\[ \text{TBX6 and} \]

\[ \text{BRA, the loss of} \]

\[ \text{WNT3A which plays a role in proliferation of} \]

\[ \text{the progenitor population, and the expanded domain of expression} \]

\[ \text{of the neural marker} \]

\[ \text{SOX2 suggests that the progenitor population has been critically jeopardized by forced differentiation} \]

\[ \text{towards a neural cell fate. Analysis of total cell numbers by} \]

\[ \text{Total cell numbers by TO-} \]

\[ \text{PR-3 Iodide labeling of the TBM posterior to the hindgut} \]

\[ \text{revealed Araucana} \]

\[ \text{have consistently fewer total cells than} \]

\[ \text{controls, and this difference was statistically significant by HH19-} \]

\[ 20 (p<0.05, \text{Fig. 8I). To examine potential drivers of the change in} \]

\[ \text{overall cell numbers we compared the levels of proliferation and} \]

\[ \text{apoptosis in the tailbud.} \]

\[ \text{Quantification by image analysis showed that proliferation was} \]

\[ \text{similar between control and Araucana} \]

\[ \text{TBM up to HH15 (Fig. 8A–B). After this a dramatic increase in proliferating cells} \]

\[ \text{was observed, lack of} \]

\[ \text{RALDH2, expression were observed, lack of CYP26A1 in} \]

\[ \text{Araucana} \]

\[ \text{could lead to increased levels of RA within the} \]

\[ \text{progenitor mesenchyme. However, if levels of RA in the tail were} \]

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\[ \text{Araucana} \]

\[ \text{tails have reduced proliferation and} \]

\[ \text{increased apoptosis} \]

\[ \text{The premature downregulation of mesodermal markers} \]

\[ \text{TBX6 and} \]

\[ \text{BRA, the loss of} \]

\[ \text{WNT3A which plays a role in proliferation of} \]

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\[ \text{Quantification by image analysis showed that proliferation was} \]

\[ \text{similar between control and Araucana} \]

\[ \text{TBM up to HH15 (Fig. 8A–B). After this a dramatic increase in proliferating cells} \]

which encodes a dehydrogenase involved in endogenous production of RA [66]. Expression of RALDH2 in the anterior PSM and the newly formed somites of AraucanaRp embryos appeared normal compared to the pattern displayed in control embryos at HH10 (Fig. 7A–B). Therefore, there does not appear to be a posterior shift in the production of RA in the tail. However, because of the reduced distance between the rostral PSM and tip of the tail in AraucanaRp embryos, RA is likely able to influence cells closer to the tip of the tail at earlier stages than normal.
Figure 7. ISH expression pattern of RALDH2, CYP26A1, WNT3A and FGF8 during tail development. RALDH2 expression in control (A) and Araucana<sup>Rp</sup> (B) embryos at HH18. Note in Araucana<sup>Rp</sup> the truncated tail at HH18 coupled with the close expression of RALDH2 in the formed somites. Posterior-most expression of RALDH2 is marked with arrowhead. CYP26A1 expression in control (C) and Araucana<sup>Rp</sup> (D) embryos at HH16. Note the lack of expression in Araucana<sup>Rp</sup> tailbud (arrowhead). WNT3A expression in control (E,G) and Araucana<sup>Rp</sup> (F,H) embryos from HH17-18. Note lack of expression in tailbud somites in Araucana<sup>Rp</sup>. FGF8 expression in control (I,K) and Araucana<sup>Rp</sup> (J,L) embryos from HH stages 17–18. Note the downregulation of expression in tailbud beginning at HH17 in Araucana<sup>Rp</sup> (arrowheads). Insets are transverse sections at level of tailbud. Anterior is top in whole mount images. Dorsal is top in transverse section insets. Sections were taken at approximate level of black bars. doi:10.1371/journal.pone.0112364.g007

Figure 8. Role of proliferation and apoptosis in Araucana<sup>Rp</sup> tailbud development. EdU labeled proliferating cells in tailbud sagittal sections at HH15-20 in controls. (A,C,E,G) and Araucana<sup>Rp</sup> (B,D,F,H) embryo tailbud. Green is EdU labeled proliferating cells, red is TO-PRO-3 Iodide labeled nuclei. Arrows denote decreased regions of proliferation in Araucana<sup>Rp</sup> compared to controls. (I) Quantification of all TBM cells for control and Araucana<sup>Rp</sup> labeled with TO-PRO-3 Iodide. (J) Quantification of proliferating TBM cells for control and Araucana<sup>Rp</sup> labeled with EdU. TUNEL labeled apoptotic cells in tailbud sagittal sections from HH stages 15–20 of controls. (K,M,O,Q) and Araucana<sup>Rp</sup> (L,N,P,R) embryo tailbud. Red is TUNEL labeled apoptotic cells, blue is Hoechst labeled nuclei. Arrows denote increased areas of apoptosis in Araucana<sup>Rp</sup> compared to controls. (S) Quantification of apoptotic TUNEL labeled cells in control and Araucana<sup>Rp</sup> TBM. Araucana<sup>Rp</sup> have significantly more TUNEL positive cells beginning at HH17 than controls. T-test, asterisk marks p<0.05. Control-Black, Araucana<sup>Rp</sup>-Grey. Same embryo (two different sections) was used for both TUNEL and EdU labeling. Anterior is towards the top, dorsal is towards the left in all sections. doi:10.1371/journal.pone.0112364.g008
was noted at HH16 in controls, with less than half the number of proliferating cells in the AraucanaRp tailbud (Fig. 8C–D). From HH17 to HH20 the reduced levels of proliferation are statistically significant (p<0.05, Fig. 8E, H,J). One possible explanation for the reduced rate of proliferation is that the fate change away from mesoderm to neural described previously will reduce the number of progenitor cells as neural cells move out of the cell cycle [76]. Exposure of the tailbud to RA leads to increased levels of apoptosis [25,65]. Historical studies in rumpless chickens identified degenerating cells within the tailbud and morphologically the tailbud appears to have apoptotic cells in the tip of the tail (Fig. 2D,F) [28]. Therefore, we examined the levels of apoptosis within the tailbud region using TUNEL labeling of sagittal sections. Apoptosis was similar up to HH16 between control and AraucanaRp embryos (Fig. 8K–N,S). At HH17–18, however, there was a dramatic rise in apoptosis within the tailbud of AraucanaRp embryos compared to controls (Fig. 8O–P,S). The increased Apoptosis in AraucanaRp occurred within the posterior TBM and surface ectoderm, matching the same area in AraucanaRp that had decreased proliferation (Fig. 8F,P). By HH19–20, apoptosis had increased in the ventral ectodermal ridge of controls, whereas AraucanaRp apoptosis was still elevated within the remaining posterior TBM, surface ectoderm, and VER (Fig. 8Q–S). These apoptotic AraucanaRp cells appear in the same position as cells that would normally be expressing TBX6 and WNT3A (Fig. 8R,P,R). Interestingly, the pattern of apoptosis seen in AraucanaRp matches that observed by Tenin and coworkers in rumpless chickens identified apoptosis [25,65]. Historical studies in rumpless chickens identified degenerating cells within the tailbud and morphologically the apoptosis [25,65].

Discussion

The Araucana rumpless phenotype results from a failure to form the most posterior axial somites. Two candidate mutations proximal to the genes IRX1 and IRX2 have been identified as associated with the rumpless phenotype. AraucanaRp exhibit a gain-of-function of both IRX1 and IRX2 genes within the tailbud during the tail organizer stage. This gain-of-function precedes observed changes in mesodermal and neural cell specification, maintenance and proliferation of the progenitor population, and regulation of the RA pathway.

Our results indicate that the AraucanaRp causative mutation is one of two (or both) SNPs within the proximal regulatory region of IRX1 and IRX2. Tena and colleagues found that the 3D architecture of IRX1 and IRX2 bring the promoters into close proximity allowing them to share enhancers [55]. The dual misexpression of IRX1 and IRX2 supports this co-regulation model in which the AraucanaRp phenotype results from a gain-of-function mutation in the regulatory region of the IRX1 and IRX2 genes. Until further functional testing is done the nature of the causative mutation is speculative, but a mutation could act by removing regionally specific repression/silencing of IRX1 and IRX2 within the tailbud, or by creating a new CNH specific enhancer. Importantly, the misexpression of IRX1 and IRX2 only occurs post gastrulation at the beginning of the tailbud stages, indicating that the upstream trigger may hold a key to understanding the change between primary and secondary body formation.

The misexpression of IRX1 and IRX2 within the tailbud precedes all observed genetic and morphological changes. The iroquois genes encode homeodomain-containing transcription factors within the TALE (three amino acid loop extension) family and are involved in proneural fate and patterning through transcriptional repression of neural antagonists [33,60,77,78,79]. Given the role of iroquois genes in specifying neural identity, we hypothesized that IRX gene misexpression at the level of the CNH, which is bipotential in chickens, and in the tailbud mesenchyme, a population that has been shown to be bipotential in other vertebrate models including mice and zebrafish, would disrupt the delicate balance between progenitor cell maintenance and mesoderm/neural specification, potentially through the repression of neural antagonists such as TBX6 [6,8,9,10,25,70]. Indeed, immediately upon misexpression of IRX1 and IRX2 there is a cascade of gene disruption; immediate downregulation of TBX6 quickly followed by downregulation of BRA, indicating loss of mesoderm identity and an arrest in mesoderm specification [9,10,11,17,19], loss of CYP26A1 that is required to degrade RA (protecting progenitor cells from differentiation and apoptosis) [26,66,67], upregulation of the neural marker SOX2 [9], and concomitant loss of WNT3A and FGFR8 that are required for maintenance and proliferation of the progenitor population (Fig. 9) [16,21,64,68,69,72]. Therefore, we propose a model where proneural gene misexpression overrides the balance of factors within the bipotential progenitor population, steering presumptive mesodermal cells toward the neural lineage.

Morphological changes in the shape of the AraucanaRp embryo tail are evident as early as HH16 and coincide with the downregulation of the mesodermal markers TBX6 and BRA (T in mice), the loss of which leads to axial truncation [11,17]. Tbx6 knockout in mice leads to a loss of mesodermal specification, and the upregulation of the neural marker Sox2 within ectopic neural tubes [10,11]. Tbx6 indirectly regulates Sox2 through repression of its N1 enhancer, making it both necessary to repress neural fate as well as push cells towards a mesodermal fate [9]. The downregulation of TBX6 expression in AraucanaRp is the first observed change in gene expression following the misexpression of IRX1 and IRX2. TBX6 expression is initially lost within the tailbud, not affecting more anterior, previously specified TBX6-positive cells. At the anterior boundary of the PSM the cyclic formation of somites continued unaffected, consuming the specified PSM. When these remaining TBX6 positive cells were incorporated into the most recent somite, somitogenesis arrested prematurely as no further PSM has been specified. BRA is required for proper mesoderm cell fate and in its absence embryos fail to form the proper mesoderm structures [18,80,81,82]. T homozygote knockout mice embryos fail to form both somites and the notochord, with heterozygotes forming a truncated axis [18,81]. In AraucanaRp embryos, notochord expression is maintained, but is absent from tailbud cells by HH17. Interestingly, the downregulation of BRA in AraucanaRp resembles zebrafish no-tail mutants (ntl, T ortholog), as well as zebrafish treated with RA, which downregulates ntl expression [70,82]. Importantly, as ntl both activates cyp26a1 and functions within an autoregulatory loop with wnt1, the loss of expression of any of the three genes is sufficient to cause axis truncation [82,83]. Similarly, the loss of expression of BRA in AraucanaRp could explain the downregulation of CYP26A1 observed at HH16, as well as the downregulation of WNT3A at HH17. Without TBX6 to repress a neural fate in AraucanaRp embryos, as well as both TBX6 and BRA to promote a preonotic mesoderm fate, cells that would normally form PSM instead form ectopic neural tissue, or remain in an undifferentiated transition state. Neural cells then leave the progenitor population to join the secondary neural tube (medullary cord), reducing the available progenitor pool and starving the paraxial mesodermal of additional cells required for ongoing somite production.

During somitogenesis, the posterior region of the vertebrate embryo including the PSM is exposed to RA expressed from the somites and FGF8 expressed from the tailbud, generating two opposing gradients [64,66,84,85,86]. The tailbud progenitor
population is protected from the effects of RA by the action of CYP26A1, which metabolizes RA [20,66]. One of the first events following IRX1/IRX2 misexpression is the loss of CYP26A1 within the tailbud, though whether the downregulation of CYP26A1 is a direct consequence of the ectopic IRX1/IRX2 expression is still unknown. In the tailbud of Araucana Rp, without the protection of CYP26A1 we would predict that the levels of RA would be increased, although we have not directly shown this. However, data from chicken and mice studies show that ectopic RA leads to the downregulation of WNT3A and FGF8, axis truncation, and ectopic neural tissue, which is precisely what we observed in Araucana Rp embryos [24,25,64,65,74].

Based on our results changes in proliferation and apoptosis occur following the initial morphological changes of Araucana Rp embryos at HH16. As cells are constantly moving from the TBM to form the PSM and then somites, a constant supply of new cells is required. Controls display a spike in proliferation at HH16, followed by a declining trend in proliferation. The number of TBM cells as labeled by TO-PRO-3 Iodide in controls appears relatively stable through these stages, indicating that proliferation may not be the only contributor to the cells of the TBM. Cells that migrate through the ventral ectodermal ridge into the TBM are most likely helping to maintain the TBM population in controls [22]. Araucana Rp do not display the spike in proliferation seen in controls at HH16, with the number of proliferating cells continuously decreasing. A significant decrease in proliferation within the TBM occurs in Araucana Rp at HH17-18, which coincides spatiotemporally with the downregulation of FGF8 and WNT3A. We are unable to determine from these results whether the downregulation of FGF8 and/or WNT3A directly causes the decrease in proliferation. However, studies in mice have shown that Wnt3a plays a role in the generation and proliferation of cells within the tailbud, and loss of Wnt3a signaling leads to axis truncation and formation of ectopic neural tissue [16,68,69]. This suggests that WNT3A is required for proper patterning of presumptive presomitic mesoderm cells and without WNT3A there is an expansion of neural tissue, as observed by the ectopic Wnt3a signaling in Araucana Rp embryos [23,24,65,74].

Furthermore, exposure to ectopic RA leads to downregulation of WNT3A and apoptosis in the tailbud [24,65,74]. It is therefore likely that in Araucana Rp embryos the apoptotic event that is triggered at HH17-18 results from exposure to increased levels of RA within the tailbud. Thus, changes in both apoptosis and proliferation do not appear to be the initial cause of changes in the morphology of the Araucana Rp phenotype, but rather occur following the initial changes in gene expression within the TBM. As proliferation decreases, and apoptosis increases, the diminution of the TBM ensues, leading to a diminished progenitor population in Araucana Rp embryos by HH19-20. As cells from this progenitor pool populate both the neural tube and PSM, Araucana Rp are unable to contribute enough cells to the PSM to continue axis elongation, as there is both a decrease in the number of progenitor cells, and an increase in cells forming neural tissue.

In conclusion, we have provided evidence that a novel gain of function is responsible for the Araucana rumpless phenotype. Hence, the Araucana rumpless mutant (Rp) is separate from the spontaneous mutations that cause the lack of a tail in chickens [88]. This study has highlighted the fine control required to maintain axis elongation and has added additional evidence that avian tailbud cells are bipotential, continuing to make germ layer decisions between neural and mesoderm post gastrulation similar to mice and zebrafish [8,70]. Considering that mutants such as T and Wnt3a mice are a loss of function, it was surprising that a
similar phenotype occurred in Araucana as a gain of function. These results provide insight into a novel developmental mechanism controlling the termination of axis elongation and therefore total axis length. Future studies are required to better understand how the Araucana gain of function mutation drives expression to unbalance the bipotential fate, and whether the same mechanism can control somite numbers in other organisms.

Supporting Information

Table S1 Number of reads, bases, coverage, SNPs, and INDELS for each Araucana following WGS.

Table S2 Complete list of 298 unique small variants found from WGS.

References

1. Schoenwolf GC (1979) Histological and ultrastructural observations of tail bud formation in the chick embryo. The Anatomical record 193: 131–140.
2. Schoenwolf GC (1981) Morphogenetic processes in the remodeling of the tail region of the chick embryo. Anatomy and Embryology 162: 183–197.
3. Catala M, Teillet M-A, Le Douarin NM (1995) Organization and development of the tail bud analyzed with the quail-chick chimera system. Mechanisms of Development 49: 51–65.
4. Cambray N, Wilson V (2002) Axial progenitors with extensive potency are localised to the mouse chordoneural hinge. Development 129: 4853–4866.
5. Cambray N, Wilson V (2007) Two distinct sources for a population of maturing axial progenitors. Development 134: 2829–2841.
6. McGrew MJ, Sherman A, Lillico SG, Ellard FM, Radcliffe PA, et al. (2008) Localised axial progenitor cell populations in the avian tail bud are not committed to a posterior Hox identity. Development 135: 2289–2299.
7. Wilson V, Olivera-Martinez I, Storey KG (2009) Stem cells, signals and vertebrate body axis extension. Development 136: 1591–1604.
8. Tzouanacou E, Wegener A, Wymeersch EJ, Wilson V, Nicolas J-F (2009) Redefining the Progression of Lineage Segregations during Mammalian Embryogenesis by Clonal Analysis. Developmental Cell 17: 356–376.
9. Gomez T, Uchikawa M, Yoshida M, Bell DM, Lovell-Badge R, et al. (2011) Tbx5-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells. Nature 474: 390–398.
10. Noszatkins S, Ferrer-Vaquero A, Concepcion D, Papasannou VE, Hadjantonakis AK (2012) Interaction of Wnt3a, Msg1 and Tbx6 in neural versus paraxial mesoderm lineage commitment and paraxial mesoderm differentiation in the mouse embryo. Dev Biol 367: 1–14.
11. Chapman DL, Papiasannou VE, Habjanto- nakis AK (1998) Three neural tubes in mouse embryos with mutations in the T-box gene Tbx6. Nature 391: 695–697.
12. Brand-Saberi B, Christ B (2000) Evolution and development of distinct cell lineages derived from somites.Curr Top Dev Biol 48: 1–42.
13. Dequeant M-L, Pourquié O (2008) Segmental patterning of the vertebral embryonic axis. Nat Rev Genet 9: 370–382.
14. Gomeza F, Oshikub EM, Wunderlich J, Baumann D, Lewis J, et al. (2008) Control of segment number in vertebrate embryos. Nature 454: 335–339.
15. Richardson MK, Allen SP, Wright GM, Raynald A, Hanken J (1999) Somite number and vertebrate evolution. Development 125: 151–160.
16. Greco TL, Takada S, Newhouse MM, McMahon JA, Mcmahon AP, et al. (1996) Analysis of the vestigial tail mutation demonstrates that Wnt-3a gene dosage regulates mouse axial development. Genes & Development 10: 313–324.
17. Yamaguchi TP, Takada S, Yoshikawa Y, Wu N, McMahon AP (1999) T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. Genes & Development 13: 3185–3190.
18. Chedey P (1935) Development of the short-tailed mutant in the house mouse. Journal of Experimental Zoology 70: 429–459.
19. Herrmann RG, Labiet S, Pouatka A, King TR, Lebrach H (1990) Cloning of the T gene required in mesoderm formation in the mouse. Nature 345: 617–622.
20. Abu-Abed S, Dolle P, Metayer D, Beckett R, Chambon P, et al. (2001) The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. Genes & Development 15: 236–240.
21. Ciruna B, Rossant J (2001) FGF Signaling Regulates Mesoderm Cell Fate Specification and Morphogenetic Movement at the Primitive Streak. Developmental Cell 1: 37–49.
22. Ohita S, Suzuki K, Tachibana K, Tanaka H, Yamada G (2007) Cessation of gastrulation is triggered by suppression of epithelial-mesenchymal transition at the ventral ectodermal ridge. Development 134: 4315–4324.
23. Mills CL, Bellairs R (1989) Mitosis and cell death in the tail of the chick embryo. Anatomy and Embryology 180: 301–308.
24. Tenen G, Wright D, Fejerskotz Z, Bone R, McGrew M, et al. (2010) The chick somatogenesis oscillator is arrested before all paraxial mesoderm is segmented into somites. BMC Developmental Biology 10: 24.
25. Olivera-Martinez I, Harada H, Halley PA, Storey KG (2012) Loss of FGF-Dependent Mesoderm Identity and Rise of Endogenous Retinoid Signalling Determine Cessation of Body Axis Elongation. PLoS Biol 10: e1001413.
26. Dunn LG, Landauer W (1981) The Genesis of the rumples and ear-tufted traits of the Araucana chicken. PLoS ONE 7: e49074.
27. Hamburger V, Hamilton LH (1951) A series of normal stages in the development of the chick embryo. Journal of Morphology 88: 49–92.
28. Yamazaki Y, Yuguichi M, Kihota S, Iosaka K (2011) Whole-mount bone and cartilage staining of chick embryos with minimal decalcification. Biotechne & Histochemistry 86: 351–356.
29. Wood JL, Hughes AJ, Mercer KJ, Chapman SC (2010) Analysis of chick (Gallus gallus) middle ear columella formation. BMC Dev Biol 10: 16.
30. Chapman SC, Schubert FR, Schoenwolf GC, Lumsden A (2002) Analysis of Spatial and Temporal Gene Expression Patterns in Bistatuia and Gastraula Stage Chick Embryos. Developmental Biology 245: 187–199.
31. McDonald LA, Gerrelli D, Fok Y, Hurst LD, Tickle C (2010) Comparison of Iroquois gene expression in limbs/fins of vertebrate embryos. J Anat 216: 603–614.
32. Quinlan R, Gale E, Maden M, Graham A (2002) Deficits in the posterior pharyngeal endoderm in the absence of retinois. Dev Dyn 225: 54–60.
33. Chapman SC, Brown R, Lees L, Schoenwolf GC, Lumsden A (2004) Expression analysis of chick Wnt and frizzled genes and selected inhibitors in early chick embryo development. Dev Dyn 229: 686–676.
34. Warren M, Puskarczyk K, Chapman SC (2009) Chick embryo proliferation studies using EdU labeling. Developmental Dynamics 238: 944–949.
35. Lohse M, Bolger AM, Nagel A, Fernie AR, Lunn JE, et al. (2012) RobinNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. Nucleic Acids Res 40: W622–627.
36. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Methods 9: 357–359.
37. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. (2009) The Sequence Alignment/Map format and SAMtools. Nat Methods 9: 357–359.
38. Ciruna B, Rossant J (2001) FGF Signaling Regulates Mesoderm Cell Fate Specification and Morphogenetic Movement at the Primitive Streak. Developmental Cell 1: 37–49.
39. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, et al. (2009) The Sequence Alignment/Map format and SAM tools. Bioinformatics 25: 2078–2079.
40. Consortium ICM (2004) A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. Nature 432: 717–722.
41. Herrmann RG (1991) Expression pattern of the Brachyury gene in whole-mount TWis/TWis mutant embryos. Development 113: 913–917.
42. Chapman DL, Aguihek I, Hancock S, Silver LM, Papasannou VE (1996) Twist is a Mouse T-Box Gene. Implication of Paraxial Mesoderm Formation at Gastrulation. Developmental Biology 180: 534–542.
43. White PH, Farkas DR, Mccadden EJ, Chapman DL (2003) Defective somite patterning in mouse embryos with reduced levels of Tbx6. Development 130: 1501–1509.
44. Buchberger A, Seidl K, Klein C, Eberhardt H, Arnold HH (1998) Meso-1, a novel HHLH transcription factor, is involved in somite formation in chicken embryos. Dev Biol 199: 201–215.
57. López-Sánchez C, Bartulos O, Martínez-Campos E, Ganán C, Valenciano AI, Gomez-Skarmeta JL, Modolell J (2002) Iroquois genes: genomic organization and function in vertebrate neural development. Development 128: 2847–2855.

58. Brott BK, Sokol SY (2005) Frodo proteins: modulators of Wnt signaling in vertebrate development. Differentiation 73: 323–329.

59. Waxman JS, Hocking AM, Stock CL, Moon RT (2004) Zebrafish Dapper1 and Dapper2 play distinct roles in Wnt-mediated developmental processes. Development 131: 5909–5921.

60. Alcover LE, Winterbottom FL, Jorge EC, Rodrigues Soberia D, Xavier-Neto J, et al. (2009) Chicken dapper genes are versatile markers for mesodermal tissues, embryonic muscle stem cells, neural crest cells, and neurogenic placodes. Dev Dyn 238: 1166–1178.

61. Cavodeassi F, Modolell J, Gomez-Skarmeta JL (2001) The Iroquois family of genes: from body building to neural patterning. Development 128: 2847–2855.

62. Rex M, Orme A, Uwanogho D, Tointon K, Wigmore PM, et al. (1997) Iroquois, a novel set of genes with multiple domains whose expression changes reflect the boundaries of embryonic domains. Development 124: 2125–2135.

63. Goldbeter A, Gonze D, Pourquié O (2007) Sharp developmental thresholds and emergent wavefront activity that controls somitogenesis. Proc Natl Acad Sci U S A 104: 12075–12080.

64. Yasuhiko Y, Haraguchi S, Kitajima S, Takahashi Y, Kanno J, et al. (2006) Dynamic expression of chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue. Dev Dyn 209: 323–332.

65. Shum ASW, Poon LLM, Tang WWT, Koide T, Chan BWH, et al. (1999) Tyrosine hydroxylase is expressed during early heart development and downregulates Bmp4. Development 126: 551–560.

66. Shum ASW, Poon LLM, Tang WWT, Koide T, Chan BWH, et al. (1999) Tyrosine hydroxylase is expressed during early heart development and downregulates Bmp4. Development 126: 551–560.

67. Sakai Y, Meno C, Fujii H, Nishino J, Shiratori H, et al. (2001) The retinoic acid-inducing enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterior-posterior axis within the mouse embryo. Genes & Development 15: 213–225.

68. Takada S, Stark KL, Shea MJ, Vasileva G, McMahon JA, et al. (1994) Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev 8: 174–189.

69. Yoshikawa Y, Fujimori T, McMahon AP, Takada S (1997) Evidence that absence of Wnt-3a signaling promotes neuralization instead of paraxial mesoderm development in the mouse. Dev Biol 183: 234–242.

70. Martin BL, Kimelman D (2012) Canonical Wnt Signaling Dynamically Controls Multiple Stem Cell Fate Decisions during Vertebrate Body Formation. Developmental Cell 22: 223–232.

71. Aubelh A, Welsch C, Brand-Saberi B, Kemler R, Gessler A, et al. (2003) Wnt3a Plays a Major Role in the Segmentation Clock Controlling Somitogenesis. Developmental Cell 4: 395–406.

72. Olivera-Martinez I, Storey KG (2007) Wnt signals provide a timing mechanism for the FGF-retinoic differentiation switch during vertebrate body axis extension. Development 134: 2125–2135.

73. Wahl MB, Deng C, Lewandoski M, Pourquié O (2007) FGF signaling acts upstream of the NOTCH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. Development 134: 4033–4041.

74. Iulianella A, Beckert B, Pekovich M, Lohnes D (1999) A Molecular Basis for Retinoic Acid-Induced Axial Truncation. Developmental Biology 205: 53–48.

75. Shindo K, Takashashi Y (2011) Secondary neuralization: Fate-mapping and gene manipulation of the neural tube in tail bud. Development, Growth & Differentiation 53: 401–410.

76. Shimokita E, Takashashi Y (2011) Secondary neuralization: Fate-mapping and gene manipulation of the neural tube in tail bud. Development, Growth & Differentiation 53: 401–410.

77. Martin BL, Kimelman D (2010) Brachyury establishes the embryonic mesodermal progenitor niche. Genes Dev 24: 2778–2783.

78. Rodriguez-Seguel E, Alarcon P, Gomez-Skarmeta JL (2009) The Xenopus irx genes are essential for neural patterning and define the border between prethalamus and thalamus through mutual antagonism with the anterior repressors Fezf and Arc. Dev Biol 329: 253–260.

79. Gomez-Skarmeta JL, de la Calle-Mustienes E, Modolell J (2001) The Iroquois family of genes: from body building to neural patterning. Development 128: 2847–2855.

80. Showell C, Binder O, Conlon FL (2004) T-box genes in early embryogenesis. Differentiation 53: 1–8.

81. Dunn LC (1925) The inheritance of rumplessness in the domestic fowl. Journal of Heredity 16: 127–134.