**Loss of FGF-Dependent Mesoderm Identity and Rise of Endogenous Retinoid Signalling Determine Cessation of Body Axis Elongation**

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**Abstract**

The endogenous mechanism that determines vertebrate body length is unknown but must involve loss of chordo-neural-hinge (CNH)/axial stem cells and mesoderm progenitors in the tailbud. In early embryos, Fibroblast growth factor (FGF) maintains a cell pool that progressively generates the body and differentiation onset is driven by retinoid repression of FGF signalling. This raises the possibility that FGF maintains key tailbud cell populations and that rising retinoid activity underlies cessation of body axis elongation. Here we show that sudden loss of the mesodermal gene (Brachyury) from CNH and the mesoderm progenitor domain correlates with FGF signalling decline in the late chick tailbud. This is accompanied by expansion of neural gene expression and a similar change in cell fate markers is apparent in the human tailbud. Fate mapping of chick tailbud further revealed that spread of neural gene expression results from continued ingestion of CNH-derived cells into the position of the mesoderm progenitor domain. Using gain and loss of function approaches in vitro and in vivo, we then show that attenuation of FGF/Erk signalling mediates this loss of Brachyury upstream of Wnt signalling, while high-level FGF maintains Brachyury and can induce ectopic CNH-like cell foci. We further demonstrate a rise in endogenous retinoid signalling in the tailbud and show that here FGF no longer opposes retinoid synthesis and activity. Furthermore, reduction of retinoid signalling at late stages elevated FGF activity and ectopically maintained mesodermal gene expression, implicating endogenous retinoid signalling in loss of mesoderm identity. Finally, axis termination is concluded by local cell death, which is reduced by blocking retinoid signalling, but involves an FGFR-independent mechanism. We propose that cessation of body elongation involves loss of FGF-dependent mesoderm identity in late stage tailbud and provide evidence that rising endogenous retinoid activity mediates this step and ultimately promotes cell death in chick tailbud.

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**Introduction**

Cells located in the tailbud of the vertebrate embryo generate the body progressively. These cell populations include axial stem cells in the chordoneural hinge (CNH, classically defined as caudal-most ventral neural tissue and distal notochord) that contribute to notochord, somites, and ventral neural tube in a self-renewing manner [1–3] and more caudally located somitic mesoderm progenitors, which have a limited self-renewing ability (Figure 1A) [4,5]. Extrinsic signals, including Wnt and FGF, are required for continued body axis elongation in the early embryo (reviewed in [2]), and this process relies on the regulated differentiation of newly generated cells as they exit the tail end. At a specific point, however, body axis elongation ceases and this must involve the regulated differentiation and/or loss of axial stem and mesoderm progenitor cells.

Changes in a number of signalling pathways can induce axial truncations in the early embryo, although how they act on specific cell populations in the later forming tailbud has not been explored. Exposure to exogenous retinoic acid (RA) can arrest body elongation [6,7] by inhibiting expression of Wnt3a [7–9]. A critical level of bone morphogenetic protein signalling is also required for normal axis elongation; loss of the bone morphogenetic protein antagonist Noggin or Noggin overexpression both generate a truncation phenotype, reviewed in [2]. Loss of FGF signalling also leads to body axis truncation at early, pre-tailbud...
The mechanism that determines body length is unknown but likely operates at the elongating tail end of vertebrate embryos. In the early embryo, fibroblast growth factor (FGF) signalling maintains a proliferative pool of cells in the tailbud that progressively generates the body. It also protects these cells from the differentiating influence of retinoic acid, which is produced by the maturing mesoderm tissues of the extending body. We show here, in the chick embryo, that the “endgame”—that is, the termination of body axis elongation—comes when the mesodermal gene brachyury is suddenly lost from axial stem cell population and presumptive mesoderm cells in the tailbud late in development. Using gain- and loss-of-function approaches, we demonstrate that this step is mediated by loss of FGF signalling. We present evidence that this is due to rising retinoid signalling in the tailbud and that FGF signalling in the tailbud no longer opposes retinoid synthesis and activity. Finally, we reveal that these events are followed by local cell death in the tailbud, which can be reduced by the attenuation of retinoid signalling but involves a mechanism that is independent of FGF signalling via its usual receptor. We propose that cessation of body elongation involves loss of FGF-dependent mesoderm identity in the late tailbud and that this is mediated by rising endogenous retinoid activity, which ultimately promotes cell death in the chick tailbud.

**Author Summary**

The mechanism that determines body length is unknown but likely operates at the elongating tail end of vertebrate embryos. In the early embryo, fibroblast growth factor (FGF) signalling maintains a proliferative pool of cells in the tailbud that progressively generates the body. It also protects these cells from the differentiating influence of retinoic acid, which is produced by the maturing mesoderm tissues of the extending body. We show here, in the chick embryo, that the “endgame”—that is, the termination of body axis elongation—comes when the mesodermal gene brachyury is suddenly lost from axial stem cell population and presumptive mesoderm cells in the tailbud late in development. Using gain- and loss-of-function approaches, we demonstrate that this step is mediated by loss of FGF signalling. We present evidence that this is due to rising retinoid signalling in the tailbud and that FGF signalling in the tailbud no longer opposes retinoid synthesis and activity. Finally, we reveal that these events are followed by local cell death in the tailbud, which can be reduced by the attenuation of retinoid signalling but involves a mechanism that is independent of FGF signalling via its usual receptor. We propose that cessation of body elongation involves loss of FGF-dependent mesoderm identity in the late tailbud and that this is mediated by rising endogenous retinoid activity, which ultimately promotes cell death in the chick tailbud.

**Results**

**FGF Signalling Persists in the Tailbud and Declines Prior to Axis Elongation Arrest**

The expression patterns of key FGF pathway genes were examined in detail in distinct tailbud cell populations (see Figure 1A) at stages of tailbud formation and maturation in the chick (Hamburger and Hamilton [HH] stage 16/17 to HH27 [36]). The FGF ligands Fgf8 and Fgf4 were strongly expressed in the tailbud but declined in the last 2 days of axis elongation from HH22 (day 4) to HH26/7 (day 6) (Figure 1B–E’ and Figure 1F’–1I’) (with the segmentation of the paraxial mesoderm ceasing at HH24 [15,16]). Initially, Fgf8 is expressed in the caudal-most neural tube and distal notochord, which together form the chordoneural hinge (CNH) and in the surrounding mesoderm progenitors (Figure 1C’) and is downregulated by HH24/25 in mesoderm progenitors, neural CNH, and most notochord CNH (Figure 1D’, 1D’’). Fgf4 is restricted to mesoderm progenitors at all stages in the tailbud (Figure 1G–1H’ and is similarly lost as this elongation as indicated by the T-mouse mutant phenotype [23,24]. In zebrafish, retinoic acid represses Bra/Ntl, which normally protects mesoderm progenitors in the tailbud from retinoid signalling by inducing expression of Cyp26a [25]. However, it is not clear that these regulatory relationships are conserved in higher vertebrates, as caudal Cyp26a expression is dependent on FGF signalling in chick and mouse embryos [13,26], while in zebrafish neither Cyp26a nor Bra/Ntl require FGF [25]. It is also worth noting that different enhancers control the expression of Bra in distinct domains in the notochord and paraxial mesoderm [27,28] and that little is known about how Bra expression is regulated in distinct cell populations within the mature tailbud nor how these might be influenced by retinoid signalling.

Exogenous RA and loss of Wnt3a can induce programmed cell death in the tailbud, however such signalling events do not always elicit apoptosis when the body axis is truncated [18,29]. It is not known whether endogenous RA signalling acts in the tailbud by promoting programmed cell death nor if this takes place in specific cell populations at key times within the maturing tailbud. Intriguingly, an imbalance of retinoid, Wnt3a, or FGF signalling can also alter the neural versus mesodermal cell fate decision in caudal regions of the early embryo [8,9,29–34]. Rising endogenous RA activity in the late tailbud might therefore act by altering signals that maintain the axial stem cell niche and/or mesoderm progenitors, resulting in loss of multipotency and diversion towards neural fate.

Although many gene regulatory relationships are conserved between vertebrate embryos, the development of caudal-motor structures seems to differ; in humans, this appears to be closest to that observed in the chick embryo: both undergo secondary neurulation and caudal regression and lack the extended tail characteristic of the mouse. Indeed, recent work has shown that the chick but not the mouse tailbud is an endogenous source of RA [17,35], raising the possibility that distinct mechanisms may operate in different species. Here we address the mechanism of body axis termination in the chick by tracking FGF and retinoid signalling dynamics in specific tailbud cell populations and correlate this with cell movements and cell specification changes as elongation ceases. Using in vivo and in vitro approaches we demonstrate that continued generation of mesoderm along the body axis, as indicated by Brachyury expression, depends on FGF signalling and show that it is attenuated by rising retinoid signalling, which also eventually promotes cell death.
tissue matures (Figure 1F–1H). The expression pattern of the FGF/Erk feedback antagonist Sprouty2 (Spry2) serves as a reporter for FGF activity [37] and is detected in caudal neural tube, mesoderm progenitors, and CNH at all stages to HH22 (Figure 1J–K) but is lost from the neural CNH and reduced in the mesoderm progenitors by HH24 (e.g., compare Figure 1K–1K to 1L–1L). Transcripts for Fgf pathway ligands and Spry2 are then lost completely from the tailbud by HH26/27 (Figure 1E, 1E, 1I, 1I and 1M, 1M). These expression patterns indicate a rapid decline in FGF signalling in neural CNH and mesoderm progenitors in the mature HH24 tailbud (summarised by Spry2 expression in Figure 1N).

Figure 1. Key tailbud cell populations and changing FGF pathway ligand expression and activity in the maturing tailbud. (A) Schematic of key tailbud tissues; chordoneural hinge (red dashed line) consists of caudal-most ventral neural tissue and distal end of notochord (black dashed line within red dashed line) and presomitic mesoderm progenitors (yellow dashed line). These cell populations are defined by position, morphology, and their fates, following mapping studies [5] and data below. In situ hybridisation during body axis elongation Fgf8 (B–E’), Fgf4 (F–I’), and Spry2 (J–N). In all figures, top rows are lateral views, bottom rows dorsal views, and sections are sagittal unless indicated otherwise. nt, neural tube; nc, notochord; s, somite. Scale bars in all figures are 100 μm.

Loss of Mesodermal and Spread of Neural Gene Expression in the Late Tailbud

In the early embryo, FGF activity is required to promote mesodermal over neural cell fate, and so declining FGF signalling might cause body axis elongation to cease if normal cell fate specification is lost in the tailbud. To examine whether cell fates change in specific cell populations in the maturing tailbud, we analysed expression of two key marker genes in detail. Strikingly, the neural progenitor marker Sox2 was found to expand into the positionally defined mesodermal progenitor domain between HH22–24, separating the distal swelling of the notochord from the mesoderm progenitors (Figure 2A–2C) (Sox2 is also detected in the remnant of the tail gut at HH20 (Figure S1), but is lost as this cell population degenerates by HH22 [38]). Concomitant with expansion of the Sox2 domain, transcripts of the early mesoderm marker Bra were lost from the position of the medial mesoderm progenitors, which surround the notochord tip (arrows in Figure 2B and 2F). In addition, Bra was downregulated in the CNH (both the distal notochord swelling and caudal-most ventral neural tube) (Figure 2F–2H). This dramatic local change in gene expression correlates with the loss of the morphologically defined contiguous notochord/CNH (asterisks in Figure 2E,2F) and the contiguous notochord/CNH (asterisks in Figure 2E,2F). These observations suggest that the axial stem cell population of the CNH and the “mesodermal progenitors” in the tailbud lose their mesoderm potential coincident with cessation of segmentation at HH24. At later stages, the broadened Sox2-expressing domain then differentiated into a multi-lumen neural tube (Figure 2I–2I).

This critical change in gene expression was further assessed at the protein level by analysis of double immunocytochemistry for Bra and Sox2 at HH22, HH24, and HH26 (Figure 2J–L). This revealed a sharp loss of Bra protein in the distal notochord tip and surrounding cells in the position of mesoderm progenitors (asterisks in Figure 2J, 2K) and a reduction in Bra in the neural CNH (defined by Sox2/Bra co-expressing cells continuous with the caudal-most neural tube), which progressively diminishes from HH24 (Figure 2J–2L). In addition, in the positionally defined mesoderm progenitor domain, Sox2 positive cells (white arrows in Figure K, K) were also apparent as well as some Sox2/Bra co-expressing cells (which here may reflect a transitional cell state as Bra is lost in this cell population).
Figure 2. Dynamics of Sox2 and Bra expression in chick and human tailbuds. (A–D) Neural progenitor marker Sox2 mRNA expands into the mesoderm progenitor domain from HH24 (arrows in A' and B') analysed in HH22–27 stages in wholemount (A–D) and in medial sagittal sections (SS) (A'–D') at low and high magnification. The mesoderm progenitor marker Brachyury (Bra) is expressed in notochord, CNH, and caudal presomitic mesoderm at HH22. Expression is lost from the distal notochord/CNH (* in E' and F') and from neural CNH and mesoderm progenitors (arrows in E' and F') from HH24–27 analysed in wholemount (E–H) and in medial sagittal sections (E'–H'). Transverse sections from rostral to caudal through HH26–27 tailbud showing termination in Sox2-expressing tissue with multiple lumens (I–I''). Immunocytochemistry for Sox2 and Bra in chick tailbuds at HH22 (J–J''). Overlap of Sox2 and Bra is confined to the neural-CNH (white dashed line), and Bra domain is continuous between notochord and CNH and mesoderm progenitors (indicated by * marking distal notochord). At HH24 (K–K''), Bra is lost from distal notochord (*) and surrounding medial mesoderm progenitors, as well as the neural CNH (which is reduced in size; white dashed line), while Sox2-expressing cells are now located between the notochord and CNH and in the “mesoderm progenitor” domain at HH26. (L–L'') Bra is detected at the caudo-lateral edge of the mesoderm progenitor domain, and the Sox2/Bra-expressing CNH is reduced still further (white dashed line). (M) Human tailbud showing Sox2 expressing cells in the neural tube and in the mesoderm progenitor domain (white dashed line) at CS12. (M') Expression of Sox2 and Bra in the CS12 tailbud; the highest levels of Bra are detected in the distal notochord (*). (N) Human tailbud showing terminal neural structures and distal notochord at CS16. (N') High-magnification view of tailbud shown in (N); Bra-expressing mesodermal progenitor cell population is no longer present at the tail tip, which terminates in a multi-lumen neural tube. Colour code for dashed lines as in Figure 1A.

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Importantly, analysis of these proteins in serial sections of the human tailbud at Carnegie Stage (CS) 12 (26–30 days, 21–29 somites, equivalent to early tailbud HH18 in terms of somite number) \( (n = 2) \) and at CS16 (37–42 days, 38–39 somites; corresponds to the end of axis elongation [39] equivalent to HH26/27) \( (n = 1) \) revealed a similar loss of CNH (Sox2/Bra cell population) and of Bra expression as this tailbud matures (Figure 2M–N). At CS12 Sox2 was detected at high levels in neural tube, but also in an adjacent discrete cell group that co-expresses Bra, which may be equivalent to the CNH (Figure 2M, 2M). Bra was also detected at high levels in notochord at CS12 (Figure 4M). Analysis of serial sections through the later CS16 human tailbud revealed that by the end of somitogenesis this was capped at its terminal end by Sox2 expressing neuroepithelium and Bra was now confined to the notochord (Figure 2N, 2N). These observations indicate that arrest of body axis elongation in chick and human is characterised by loss of Bra expression in the tailbud, leaving terminal cells with a neural character.

Fate Mapping the Late Tailbud Demonstrates Continued Cell Ingression from the CNH

To understand the contribution that cell movement might make to these changing patterns of gene expression, a series of fate mapping studies were carried out in the tailbud in ovo (Figure 3A and see Materials and Methods). Dil was used to label focal groups of \( \sim 20–40 \) cells in the caudal-most neural tube, the “neural CNH,” or the mesoderm progenitor domain at HH20 (local labelling was confirmed by observation in the whole embryo, and to ensure accurate labelling, a subset of embryos were fixed immediately and sectioned to define position of Dil labelled cells; Figure 3B–3D, Table 1). After 30 h incubation (to HH24/25), neural tube cells were found to contribute only to neural tissue (Figure 3B, 3B), while cells in the CNH contribute to neural tissue and the positionally defined mesoderm progenitor domain, presomitic mesoderm, and somites (Figure 3C–3C, Table 1). This suggests that at least some of the Sox2 positive cells that appear in this “mesoderm progenitor” territory at HH24 are derived from the CNH (Figure 3C). We also wished to ascertain whether cells present in the Bra positive mesoderm progenitor domain at HH20 (prior to the appearance of Sox2 here at HH24) later come to express Sox2. Mesoderm progenitors labelled at HH20 were therefore assessed for Sox2 expression at HH24. However, the majority of Dil-labelled cells left the mesoderm progenitor domain and contributed to pre-somitic, somitic, and/or more lateral mesoderm, and few remaining cells were in the new Sox2 expressing domain (Figure 3D, Table 1). These data (summarized in Figure 3E) therefore suggest that Sox2 expansion into the tailbud core is due to maintenance of this gene and loss of Bra in cells that have recently ingressed from the CNH.
FGF Signalling Is Required to Maintain Mesodermal Gene Expression in the Tailbud

The loss of Bra and expansion of the Sox2 expression domain into the tailbud at HH24 correlate well with the decrease in FGF signalling in the mesoderm progenitor domain and neural CNH (summarized in Figure 1N and compare Figure 1K" and 1L" with Figure 2E' and 2F'). To test whether decline in FGF signalling underlies this striking change, FGF signalling was blocked in HH19–22 tailbuds using the Fgf receptor inhibitor PD173074 or the Mitogen-activated protein kinase kinase (MEK) antagonist PD184352, which blocks downstream Erk1/2 activity. Control tailbuds exposed to DMSO alone expressed Spry2 in the mesoderm progenitor domain (Figure 4A) and Bra in the mesoderm progenitors and notochord (while Bra expression in intervening CNH/medial mesoderm progenitors was downregulated as normal) (arrow in Figure 4B); Sox2 expression extended to the tailbud tip (Figure 4C), and Tbx6L was expressed in mesoderm progenitors and extended into caudal presomitic mesoderm (Figure 4D); all gene expression patterns typical of the normal HH23–24 tailbud. Exposure to PD173074 or PD184352 inhibited expression of Spry2 (DMSO n = 1/16, PD173074 n = 14/16; DMSO n = 1/4, PD184352 n = 3/4) (Figure 4A–4A") and repressed all Bra expression except in the proximal notochord (Bra, DMSO n = 0/10, PD173074 n = 10/10; DMSO n = 0/4, PD184352 n = 6/7; Figure 4B–B") while Sox2 transcripts were more widely detected caudally in comparison with controls (Sox2, DMSO n = 1/6, PD173074 n = 4/7; DMSO n = 0/3, PD184352 n = 3/3; Figure 4C–4C"). A recent study has shown that the further T-box gene Tbx6 is required to downregulate Sox2 expression in the early mouse embryo [40]. The homologous gene Tbx6L is downregulated as the chick tailbud matures [17], and so its loss provides a potential mechanism to explain the upregulation of Sox2 in the mesoderm progenitor cell population. Importantly, blocking FGFR- or MEK-mediated signalling also inhibited Tbx6L (Tbx6L, DMSO n = 0/7, PD173074 n = 7/7;
DMSO n = 0/10, PD184352 n = 11/11; Figure 4D–4D), demonstrating that maintenance of this further mesoderm identity gene is dependent on FGFR/Erk activity. As Bra is a known direct target of Wnt signalling, we also assessed the effects of FGFR and MEK inhibition on expression of the key ligand Wnt3a. This gene is expressed in tailbud mesoderm and dorsal neural tube, and its expression was repressed by both FGFR or MEK antagonists specifically in the tailbud mesoderm (DMSO n = 0/3; PD173074 n = 8/8; DMSO n = 0/6, PD184352 n = 10/10; Figure 4E–4E*).

Finally, grafting an FGFR delivering bead into the HH20–22 tailbud induced ectopic expression of Bra (BSA bead control n = 0/7; FGFR beads n = 4/6 embryos; Figure 4F–4F**). FGFR beads were also found to elicit an ectopic patch(es) of Sox2 expression in cells close to the bead (BSA n = 0/6; FGFR n = 5/7; Figure 4G–4G*). This initially surprising result may reflect the regulation of Sox2 by distinct enhancer elements active around the organizer region (N1) and in the developing spinal cord (N4) [41]; expansion of Sox2 expression on FGFR inhibition (results above) may be indicative of spinal cord differentiation via N4 activity, while induction of Sox2 by high-level FGFR may reflect the N1 element, which is FGFR/Wnt responsive [42] and might be indicative of formation of an ectopic CNH.

To address if a CNH-like state is induced, we determined whether FGFR-induced ectopic Sox2 positive cells co-express Bra, as observed in the endogenous CNH. In most cases, ectopic co-expression was detected, suggesting that these cells have CNH character (BSA n = 0/4; Figure 4H–4H*; FGFR n = 4/6; Figure 4I–4I*). Some of the ectopic Sox2/Bra cells formed small groups, but we also detected strong Sox2 expression in Bra positive cells in mesoderm progenitor domain/presomitic mesoderm in tails with FGFR beads (Figure 4I–4I*). In 3/6 embryos, ectopic Sox2/Bra foci exhibited a polarised configuration, being flanked at either end by Sox2 or Bra only expressing cells, reminiscent of tissue organisation around the endogenous CNH (Figure 4I**–4I***). Together these findings (summarised in Figure 4J) therefore suggest that maintenance of mesoderm progenitors and the CNH depends on FGFR/Erk signalling and that reduction of such signalling leads to neural differentiation in the tailbud, while high-level FGFR activity can promote a CNH-like cell state.

Key Components of the Retinoid Pathway Are Detected in the Late Stage Tailbud

As FGFR signalling is attenuated by RA provided by somites in the early embryo, we next tested whether the RA syntheisising enzyme Raldh2 continues to be expressed in somites throughout body axis elongation and if expression of RA signalling pathway genes alter in the tailbud. Expression of Raldh2 was detected in the most recently formed somites throughout body axis elongation (Figure 5A–5D*). However, we additionally observed the appearance of domains of Raldh2 in the tailbud itself prior to elongation arrest by HH24 (also see [17] for report of whole embryo expression pattern). Tissue localisation of Raldh2 in sections revealed expression in caudal mesoderm progenitors in the tailbud by HH24 (Figure 5C–5C*). These observations suggest a means by which endogenous retinoid signalling can be locally increased in the tailbud prior to body elongation arrest. Initially transcripts for the retinoic-acid-catabolising enzyme Cyp26a were restricted to superficial ventral ectodermal cells, distal notochord, and the CNH at HH16 (Figure 5E–5E*). In contrast, by HH24, Cyp26a was lost from the distal notochord/CNH (Figure 5G–5H*), laying this cell population open to an increase in retinoid signalling. Cyp26a expression may thus protect the axial stem cells in the CNH from retinoid signalling in the early tailbud.

### Table 1. Summary data for fate map of the late tailbud.

| Tissue Labelled | n | NT | CNH | MP | Pre-Somatic Mesoderm | Somite | Lateral Mesoderm |
|----------------|---|----|-----|----|----------------------|--------|-----------------|
| Neural tube    | 8 | 8  | 1   | 0  | 0                    | 0      | 0               |
| Chordo-neural hinge | 12 | 12 | 12  | 11 | 7                    | 6      | 7               |
| Mesoderm progenitors | 11 | 0  | 9   | 9  | 9                    | 9      | 11              |

Summary of contributions of cell groups DiI labelled in HH20 neural tube (NT), chordoneural hinge (CNH), or mesoderm progenitors (MP) to tissues in and derived from the tailbud observed at HH24. n, number of embryos labelled at HH20 for each tissue.

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Analysis of RA Activity in the Maturing Tailbud

To assess the pattern of retinoid activity, we first analysed the expression of RARβ (a canonical Direct Repeat [DR] RARE (Retinoic Acid Response Element)–driven gene [43]). However, while RARβ was detected in neural tube flanked by somites at all stages, it was only weakly detected in the tailbud at HH24/25 (Figure S2). As Raldh2 is expressed in the tailbud at this time, these observations support the possibility that the RARβ (DR5) RARE does not reveal all sites of retinoid activity [44]. We therefore analysed expression of a gene with different RARE elements, as these might be recognised by distinct combinations of RAR/ RXRs [45]. *Cellular retinoic acid binding protein* (*Crabp2*) is an established RA target and its transcription is trans-activated by RARE1 and RARE2 DR motifs that are separated by one (DR1) and two (DR2) base pairs, respectively [43,45,46]. The *Crabp2* gene is expressed in early chick and mouse embryos [47–50], and here we examined its transcription in the maturing chick tailbud (Figure 5I–5L). From HH15/16, *Crabp2* transcripts are detected at low levels in the tailbud (Figure S3A–S3A*), and detailed analysis from HH19/20 to HH26 revealed that *Crabp2* then steadily encroaches on mesoderm progenitors and the CNH (Figure 5I–5L*); at HH19–22 *Crabp2* is absent from the CNH but detected in mesoderm progenitors (Figure 5I–5L*); by HH24, *Crabp2* is detected in neural/CNH (ventral caudal-most neural tube) and surrounding medial-most mesoderm progenitors, but not the distal-notochord portion of the CNH (Figure 5K–5K*); and by HH26, a small group of *Crabp2*-expressing cells are then detected in the now abrupt distal end of the notochord (Figure 5L–5L*), while transverse sections through the tail tip at this stage confirmed the multi-lumen nature of the terminating neural tube (Figure 5L*, 5L**). These expression patterns correlate well with onset of Raldh2 and downregulation of Cyp26a at HH22–24 and indicate an increase in retinoid signalling in key cell populations of the maturing tailbud (summarized in Figure 5M).

Although *Crabp2* is an established RA target in cultured cells, this has not been demonstrated in the embryo. To test this, RA-delivering beads were implanted between the caudal lateral epiblast/stem zone and presomitic mesoderm in HH9–10 chick
Figure 5. Expression of retinoid pathway genes in the maturing tailbud. Raldh2 expression in the maturing tailbud (A–D”). Arrows indicate new tailbud Raldh2 domains in the caudo-lateral mesoderm progenitors (C–C’). Cyp26a expression is downregulated in the early tailbud and is absent by HH24 (E–H”). Gradual expansion of Crabp2 transcription into chick tailbud from HH19 to HH26 in whole-mount (I–L), analysed in lateral (I’–L’) and medial (I”–L”) sagittal sections (SS) and in transverse sections (TS) of rostral (I”–I’”) and caudal (I”–I’”) tailbud. Black dashed lines indicate distal tip of notochord in (SS) and notochord in TS; red dashed lines indicate CNH at stages where this can be defined (HH19, 22, 24). Asterisk in (K”) indicates neural CNH. Summary of changes in Crabp2 expression between HH22 and HH24, indicating increasing RA activity in mesoderm progenitors and neural portion of the CNH and at edges of notochord CNH as body axis elongation ceases at HH24 (M). Bead grafting schematic (N). Expression of Crabp2 in chicken embryos grafted with DMSO bead (N”) or retinoic acid (RA) bead (N”), indicating Crabp2 increase in response to RA. Crabp2 in quail embryos raised on normal (NQ) (O) or Vitamin A–deficient diet (VADQ) (O”), demonstrating RA-dependence of Crabp2 transcription in vivo. Tailbud explant pairs (P) cultured in the presence of (P’) control DMSO or (P”) RAR/RXR antagonists shows RA dependence of Crabp2 expression in late tailbud tissue. Abbreviations as in Figure 1.

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embryos (Figure 5N). RA upregulated Crabp2 expression at the tail-end \((n = 4/5)\) compared to control DMSO beads \((n = 0/5)\), assessed after 16 h (Figure 5N–5N). In addition, tailbuds at HH20/22 treated with 100 nM RA for 24 h in vitro also demonstrated increased Crabp2 expression in comparison with DMSO-treated controls (Figure S3B–S3B). To determine whether Crabp2 transcription depends on RA signalling, Crabp2 expression was next assessed in Vitamin-A-deficient quail embryos. Crabp2 transcripts were much reduced in VAD quails \((n = 3/3)\) compared to normal quails fixed and processed in parallel \((n = 7)\); (Figure 5O–5O'). To specifically test the requirement for RA for Crabp2 expression in the tailbud, retinoid signalling was blocked using RAR/RXR antagonists; this leads to Crabp2 repression in 9/12 explant pairs (Figure 5P–5P'). In addition, tailbud explants were treated with the RA synthesis inhibitor disulfiram, and RAR/RXR antagonist delivering beads were transplanted into the HH20/21 tailbud in vivo, and in both conditions Crabp2 expression was reduced compared with DMSO-treated controls (Figure S3C, S3C', S3D and S3D'). These data demonstrate that Crabp2 provides an alternative, D1/D2-RARE-mediated mediator for retinoid signalling in vivo and that it reports increasing RA activity in the tailbud.

**Fgfs Are Repressed by RA and FGF Represses Somitic Raldh2 During Axis Elongation**

From the early tailbud (HH22) Crabp2 overlaps with Fgf-responsive domains, raising the question of whether FGF and RA signalling maintain their mutually inhibitory interactions in the tailbud. To test the regulatory relationships between these two pathways, we first exposed the prospective tailbud region at HH9–10 to exogenous RA delivered locally on a bead in vivo. This led to loss of Fgfr2 and truncation of the body axis \((n = 8/9, DMSO\) beads \(n = 0/5 \)embryos) (Figure 6A, 6B). To address the effects of RA on tailbuds at later stages we explanted and cultured tails from HH16 chick embryos for 24 h in DMSO with or without RA. In all cases, while control (DMSO exposed) tissue maintained Fgf8 expression \((n = 6)\), RA-treated tailbuds all lost Fgf8 transcripts \((n = 6/6)\) (Figure 6C–D'). To determine whether RA retains the ability to repress FGF signalling, RA was administered to HH19–22 tailbuds. After 24 h, Fgf8 and Fgf4 expression were still detected in control explants \((n = 9 and n = 8, respectively)\), but in the presence of high (10 \(\mu\)M) or low (100 nM) RA, both these genes were repressed \((Fgf8 10 \mu\)M, \(n = 9/10\); Fgf8 100 nM, \(n = 3/4\); Fgf4 10 \(\mu\)M, \(n = 3/8\); Fgf4 100 nM, \(n = 3/4\)) (Figure 6E–G'). These data show that retinoid signalling can repress FGF signalling during axis elongation and that it retains this ability in the late stage tailbud.

To test if somitic Raldh2 can be repressed by FGF signalling at early tailbud stages, pairs of HH16 trunk explants were dissected from the same embryo and either exposed to BSA vehicle control only or FGF8 (Figure 6H). After 24 h, Raldh2 is still expressed in somites and adjacent lateral plate in control explants, but in FGF8-treated explants, Raldh2 is downregulated in both domains \((n = 8/9 \)pairs) (Figure 6I). To test this regulatory relationship at later stages, pairs of HH19–22 trunk explants were cultured for 24 h. Raldh2 was still markedly reduced in all tissues in the presence of FGF8 \((n = 19/22 \)pairs) (Figure 6J, 6K, 6K'). Together these experiments indicate that throughout axis elongation caudal FGF signalling continues to repress onset of RA synthesis in forming somites and that Fgf4 and Fgf8 transcription in the tailbud remains susceptible to inhibition by RA.

**FGF No Longer Antagonises RA Signalling in the Tailbud**

FGF signalling interferes with the RA pathway in the early embryo [2,12]; however, in the early tailbud, there is an overlap between RA (Crabp2) and FGF (Sprouty) activity in mesoderm progenitors. We therefore tested whether FGF retains the ability to block RA activity in the tailbud and found that exposure to Fgf8 protein did not downregulate expression of Crabp2, in tailbud pairs (12/12) (Figure 6J, 6L, 6L'). To test whether tailbud Raldh2 is regulated by FGF, HH19–22 tailbuds were cultured for 24 h with or without FGF8. Raldh2 was detected in control \((n = 8/8 \)pairs) and also FGF8b-treated tailbud explants \((n = 7/7 \)pairs) (while control neural tube treated with FGF8b exhibited a reduction in neuron numbers, \(n = 4/4\), indicative of active FGF signalling [51]; unpublished data). To confirm that trunk and tail Raldh2 domains are regulated differently, trunk and tailbud were explanted from the same embryos (Figure 6J, 6K, 6K', 6M, 6M'). While somitic Raldh2 was inhibited \((n = 8/10 \)pairs), tailbud Raldh2 was unchanged in all cases \((n = 10 \)tailbud pairs). This failure of FGF to repress tailbud Raldh2 and Crabp2 transcription was further confirmed by local delivery of FGF4 into the late stage tailbud in vivo [Raldh2, 5/5 control BSA beads, 6/7 FGF4 beads; Crabp2, BSA beads 5/5, FGF4 beads, 5/5] (Figure 6N, 6N', 6O, 6O'). In the distinct signalling context of the tailbud, therefore, FGF no longer antagonizes retinoid synthesis or RA activity.

**Retinoid Signalling Attenuates FGF Signalling and Directs Cell Fates in the Tailbud**

We have shown that Fgf4 and Fgf8 are inhibited in the tailbud by exposure to RA. Consistent with this, we find that treatment of HH19–22 tailbuds with RA \((100 \text{ nM})\) for 24 h abolishes expression of Spry2 and additionally leads to loss of Bora, caudal expansion of Sox2, and also attenuation of Tbx6L in the mesoderm progenitor domain of the tailbud [Spry2, DMSO \(n = 0/4\), RA \(n = 4/4\) (Figure 7A–7A'); Bora, DMSO \(n = 2/11\); RA \(n = 8/14\) (Figure 7B–7B'); Sox2, DMSO \(n = 2/16\), RA \(n = 12/17\) (Figure 7C–7C'); Tbx6L, DMSO \(n = 0/7\), RA \(n = 7/9\) (Figure 7D–7D')). To test the requirement for endogenous retinoid signalling specifically in the tailbud, we further treated tailbuds with the RA synthesis inhibitor disulfiram, which reduced Crabp2 expression (Figure S3B, S3B'). This led to expansion of Spry2 and Bora expression in the region of the mesoderm progenitor domain (Spry2, DMSO \(n = 0/4\), disulfiram \(n = 4/4\) (Figure 7A', 7A'); Bora, DMSO \(n = 0/6\), disulfiram \(n = 9/11\) (Figure 7B', 7B')) while Sox2 expression was reduced caudally (Sox2, DMSO \(n = 0/11\), disulfiram \(n = 10/13\) (Figure 7C', 7C')), and Tbx6L remained strongly expressed, but did not consistently expand its domain in this timeframe (Tbx6L, DMSO \(n = 4/4\), disulfiram \(n = 4/4\) (Figure 7D', 7D')). These findings suggest that levels of endogenous RA determine FGF activity and subsequent neural versus mesoderm cell fate choice in the mesoderm progenitor domain of the tailbud.

To test whether endogenous RA activity mediates this step in an in vivo context, Vitamin A-deficient (VAD) quail embryos were examined at early tailbud stages HH11–15; retinoid deficiency is embryonic lethal due to heart defects [52,53], and this was the latest stage at which such embryos were readily available. These embryos exhibit ectopic dorsal domains of Bora and Fgf8 within the neural tube in the caudal-most region \((n = 0/4 \)normal quails, \(n = 5/6 \)VAD quails, Bora [Figure 7E–7F']; and \(n = 0/5 \)normal quails, \(n = 4/4 \)VAD quails, Fgf8, [Figure 7G–7HI']). Sox2 expression was detected throughout the neural tube in normal quails \((n = 4\), while asymmetric reduction was observed in the dorsal neural tube of some VAD embryos \((n = 2/4\) (Figure 7J–7J'). These findings indicate that endogenous RA signalling is required for the correct assignment of neural and mesodermal cell fates and for the normal downregulation of Fgf8 in the early tailbud. To test the continued involvement of retinoid signalling at the latest...
tailbud stages in vivo, we then grafted beads soaked in RAR/RXR antagonists into the tailbud at HH20–21 and cultured them for a further 24 h (Figure 7K). We first confirmed that delivery of these antagonists in vivo reduced expression of Crabp2 (Figure S3D, S3D′) (and in more rostral regions RARb, DMSO n = 0/7, RAR/RXR antagonists n = 5/6; unpublished data). We then ascertained that blocking RA signalling in this context lead to increased FGF signalling as indicated by levels of Spry2 (Spry2, DMSO n = 1/5; RAR/RXR, n = 6/6 [Figure 7L, 7L′]) and to ectopic expression of Bra in the tailbud (Bra, DMSO n = 0/8; RAR/RXR n = 3/6 [Figure 7M–7M′]). In addition, Bra was also ectopically maintained by exposure to RAR antagonist alone (Figure S4A–S4B′) controlling for potential effects on heterodimers formed by RXRs with receptors unrelated to retinoid signalling. These antagonists did not, however, elicit a consistent change in Sox2 expression (Sox2, DMSO n = 6; RAR/RXR n = 5 cases [Figure 7N–7N′]), which may reflect maintenance rather than excess levels of FGF signalling in this condition. In conclusion, in

**Figure 6. FGF and RA pathway interactions during body axis elongation.** Unlike control DMSO beads (A), retinoic acid delivering beads (B) repress Fgf8 in the early tailbud. Tailbud explanted from HH16 embryos (C) cultured in control DMSO (D) or RA (9-Cis, 10 μM) (D′) and HH19-22 tailbud explants pairs (E) cultured with DMSO (F, G) or RA (1 μM AT-RA, F; 100 nM AT-RA, G′) showing RA represses Fgf8 and Fgf4 in the maturing tailbud. RA inhibited both Fgf8 and Fgf4 in these tailbud halves at the lowest concentration of 100 nM AT-RA (n = 3/4 explant pairs for each gene). Trunk explant pairs (neural tube flanked by somites and lateral plate mesoderm) from HH16 (H) cultured with BSA vehicle control (I) or FGF8 (I′) lose Raldh2 in response to FGF8. Older, HH19-22 trunk explant pairs (J) also downregulate Raldh2 in response to FGF. BSA control (K), and FGF8 treated (K′). HH19–22 tailbud explant pairs (J) do not alter Crabp2 levels in response to FGF, control BSA (L), and FGF8 (L′). HH19–22 tailbud explant pairs do not alter Raldh2 levels in response to FGF, control BSA (M), and FGF8 (M′). Explants in (K), (K′), (M), and (M′) are taken from the same embryo, cultured and processed in parallel. In addition, neither BSA nor FGF4 delivering beads repressed expression of Raldh2 (N, N′) or Crabp2 (O, O′) expression in the tailbud in vivo. All explants are orientated as in (D). C, Caudal; R, Rostral; asterisks indicate beads.

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Figure 7. Endogenous retinoid signalling represses mesodermal and promotes neural cell fate in the tailbud. Schematic of whole tailbud explant experiment (A). Spry2 expression in vehicle control DMSO (A'), retinoic acid (RA) (A''), or retinoic acid synthesis inhibitor Disulfiram (A''') treated tailbuds. Bra expression in DMSO- (B), RA- (B'), or Disulfiram- (B'') treated tailbuds (nc, notochord; mp, mesoderm progenitors; black arrow in B indicates normal downregulation of Bra in CNH/medial mesoderm progenitors at HH24 and white arrow in B'' indicates continued expression in these tissues when RA synthesis is inhibited; Sox2 expression in DMSO- (C), RA- (C'), or Disulfiram- (C'') treated tailbuds. Tbx6L expression in DMSO- (D), RA- (D'), or Disulfiram- (D'') treated tailbuds; Quail embryos at HH14–15 were analysed for marker gene expression following development in normal (NQ) and retinoid deficient (VADQ) conditions; mesodermal marker gene Bra in normal (E, E') and retinoid deficient embryos (F, F'), where it is ectopically expressed in the dorsal neural tube (arrow). Fgf8 in normal (G, G') and retinoid deficient embryos (H, H'), where it is ectopically expressed in the dorsal neural tube (arrow). Note that the normal quail expressing Fgf8 is at a slightly later stage and is larger overall than the retinoid deficient quail, however no ectopic patches of Fgf8 are seen in normal quail neural tube (this section is also not perfectly transverse and is slightly caudal to that shown for in H'; this explains the apparent broader ventral expression of Fgf8 in the normal quail). Neural progenitor marker Sox2 in normal (I, I') and retinoid-deficient embryos, where it is downregulated in dorsal neural tube (arrow) (J, J'); (E', F', G', H', I', J') are transverse sections through the caudal neural tube that contains a lumen. Schematic of in vivo bead implantation experiment (K). Spry2 expression in the tail bud incubated with vehicle control DMSO (L) or RAR/RXR antagonists beads (L'). Bra expression with DMSO (M) or RAR/RXR antagonist (M') soaked beads. Sox2 expression with DMSO (N) or RAR/RXR antagonist (N') soaked beads (M', beads; arrow, ectopic Bra). doi:10.1371/journal.pbio.1001415.g007
this tissue and suggest that rising endogenous retinoid signalling can promote cell death in the chick tailbud via an FGF-independent mechanism.

**Discussion**

This study provides a detailed account of the cellular and molecular changes in the chordo-neural-hinge and mesoderm progenitors in the chick tailbud that underlie the normal process of body axis termination. It reveals a critical loss of mesodermal cell identity in these cell populations in the late tailbud and shows that this is due to a decline in FGFR/Erk signalling, high levels of which can promote a CNH-like cell state. We provide evidence that rising endogenous Retinoid signalling mediates FGF signalling loss and regulates the survival of CNH/axial stem cells and their tailbud derivatives.

Fate maps of the tailbud made with chick/quail chimeras or GFP expressing cells at stage HH15 (25 somites) have shown that mediately located cells defined as CNH can still give rise to both neural and mesodermal derivatives [1,5]. In agreement, we observe that later at HH22 cells located in the equivalent region express both *Bra* and *Sox2*. Moreover, our data reveal a striking loss of the mesodermal gene *Brachyury* specifically in the neural and notochord portions of the CNH and in cells located in the mesoderm progenitor domain. This is accompanied by an expansion of the neural progenitor marker *Sox2* into this “mesoderm progenitor” territory in the late (HH24) chick tailbud. Importantly, we show that the neural and notochord CNH persist and remain contiguous with the mesoderm progenitor domain at HH22 (~44 somite stage) and that cells continue to ingress from the neural CNH into the tailbud at stages leading up to HH24. These findings therefore indicate that the conditions for cessation of body axis elongation arise discretely in the late tailbud and coincide with the end of segmentation at HH24.

This cell state change is coincident with FGF signalling decline in these cell populations, and we demonstrate that blocking FGF/
Erk signalling precisely mimics this step, specifically repressing Bra in the CNH and mesoderm progenitors and leading to caudal expansion of the Sox2 expression domain. Consistent with this role for FGF signalling, a recent report analysing the spectrum of phenotypes for human syndromes generated by heterozygous constitutive activation of FGFR2 (Pfeiffer, Crouzon, and Beare-Stevenson syndromes) included an ectopic “caudal appendage” [56], suggesting conservation in humans of a mechanism that attenuates FGFR signalling for normal cessation of body axis elongation. Our finding that the maturing human tailbud also exhibits loss of Bra and terminates in a Sox2-expressing neural structure further supports conservation of the role of FGF in determining cell fates at the end of body axis elongation.

Interestingly, human tails with activated FGFR2 have a variable form, with or without vertebral [56], indicating that excess FGF signalling can generate ectopic and disorganised tail end structures. This phenotype may depend on the level of FGF activity and relate to our finding of ectopic CNH-like cells co-expressing Bra and Sox2 in response to high-level FGF delivered by beads. Our gain- and loss-of-FGF-function experiments indicate that while loss of FGF promotes neural differentiation, FGF maintains mesoderm progenitors and even higher levels may then promote the axial stem cell state of the CNH. The effects of elevated FGF and Wnt signalling may also explain the large club-end truncation phenotype of Cdx2 mis-expressing mice [57] as Cdx genes are upstream of these pathways [18,19], which need to be downregulated for segmentation and differentiation in the body axis. Similarly, long-term loss of RA (leading to maintenance of Cdx2 expression in lower vertebrates [58], the positive regulatory initiation. Indeed, it is likely that driving FGF only in the CNH exhibits loss of Bra and terminates in a Sox2-expressing neural structure further supports conservation of the role of FGF in determining cell fates at the end of body axis elongation.

Our findings are consistent with the direct regulation of Bra by FGF signalling in lower vertebrates [58], the positive regulatory loop of FGF and Wnt signalling in the early mouse embryo [22] and of Bra and Wnt3a in caudal regions in mouse and zebrafish early embryos [27,59,60]. Furthermore, mice mutant for Bra exhibit body axis truncation [23,24], and it may be important that the sudden loss of Bra/Wnt/FGF that we observe coincides with the cessation of segmentation of the presomitoc mesoderm in the chick at HH24 [15–17]. Indeed, Bra/T box proteins exert direct positive regulation on the Notch pathway ligand Delta1 and oscillations in this pathway underpin the segmentation process [61]; reviewed in [62]). The regulation of Bra by FGF/Erk signalling thus appears to be a pivotal mechanism that determines vertebrate body length by maintaining mesoderm potential and also by control of a key Notch pathway gene integral to the segmentation of the body axis.

Importantly, in vivo and in vitro assays, we show that specifically in the tailbud FGF no longer inhibits Raldh2 expression or RA activity. The downregulation of the p450 enzyme Cyp26a in the early tailbud, a known FGF-dependent gene in caudal regions in chick and mouse [15,26], further suggests that RA is no longer degraded in the chick tailbud. However, we show that RA retains the ability to repress Fgf4 and Fgf8 in the tailbud; this could be indirect via loss of Wnt3a, which maintains Fgf8 (although we also show here that FGF in turn maintains Wnt3a, as in the early mouse embryo) [10,63,64] and/or directly through binding of an RARE in the Fgf8 promoter [65,66]. This breakdown of the oppositional signalling between FGF and RA pathways and the loss of homeostatic regulation of RA turnover provide a mechanism for the steady increase in endogenous RA levels.

We present multiple lines of evidence that raising endogenous retinoid signalling then contributes to loss of FGF/Bra in the tailbud. Using Cyp26a we show that as FGF signalling declines, endogenous RA activity increases in mesoderm progenitors and the neural CNH and, eventually, in the CNH/notochord distal tip. Cyp26a transcription is well-established as an RA target in primary cells and cell lines (e.g., human skin fibroblasts [67], F9 teratocarcinoma cells [68], P19 embryonic carcinoma cells [46], and ES cells [69]). It is validated here as a reporter of RA activity in the embryo by its increase in response to exogenous RA delivered by beads or directly on explanted tailbud and its reduction in Vitamin A-deficient quails and inhibition by RA synthesis inhibitor disulfiram and by RAR/RXR antagonists in in vitro and in vivo late stage tailbuds. In these multiple retinoid deficiency assays, we then consistently find ectopic maintenance of FGF signalling and Bra, strongly suggesting a role for endogenous retinoid signalling upstream of the loss of mesodermal potential in this tissue. In addition, recent work has confirmed that the chick tailbud is a source of RA, as explanted late-stage tailbuds stimulate expression of an RARb-RARE-LacZ reporter cell line [17,70]. In the same assay, the mouse tailbud appears not to provide RA, despite also up-regulating Raldh2 [17,35]. Most recent data suggest that retinoic acid is not synthesised in the mouse tailbud [35] and so implicate a different mechanism for attenuation of FGF/Wnt signalling as elongation ceases in this animal [4]. This species difference may relate to the retention of the mouse tail, while rising retinoid signalling in the chick additionally regulates cell death and possibly the later normal process of caudal regression in chick and human [71].

Increasing levels of apoptosis were detected in the CNH and medial mesoderm progenitors after HH24. This may serve to remove remaining axial stem and mesoderm progenitor cells but appears too late to be responsible for the localised loss of Bra expression. Fate maps of the HH15 tailbud do not indicate that most terminal tissue contributes to neural tube when assessed at HH30 [1], further suggesting that terminal neural tissue identified here may also subsequently undergo apoptosis. A secondary role for cell death in the regulation of axis elongation is consistent with the phenotypes of mice in which RA levels are raised; in Cyp26a null embryos, ectopic neural tubes form at the expense of mesoderm, but cell death is not increased in the truncated body axis [29], nor are cell death patterns altered in truncations induced by Cdx mutations [18]. This suggests that endogenous cell death is induced by exposure to higher or longer duration RA and, as inhibition of FGF signalling did not increase cell death in our assays, may act via a distinct mechanism to promote this further step.

In conclusion, these data define a series of regulatory events that control the cessation of body axis elongation in the late stage chick tailbud (Figure 9). It will be important now to identify the signals that promote Raldh2 expression in the tailbud, what regulatory mechanism blocks retinoid synthesis in mouse tailbud, and the extent to which the mechanisms identified here in the chick are conserved across other vertebrate species. It will further be interesting to assess if the same signalling dynamics are deployed to arrest elongation of other axial outgrowths, such as the limb and beak that are also truncated by excess retinoic acid [72,73].
and RXR antagonists LG100815 and LG101208 (5 mM) is downregulated; consequently, RA signalling begins to rise in the tailbud. FGF signalling is required to maintain Cyp26a expression (dashed arrow) into the previous stage 20 embryonic domain, which reflects continued cell ingestion from the CNH. At this stage Crabp2 is now detected in the neural-CNH and position of the mesoderm progenitors, indicating rising retinoid signalling, which can repress FGF ligands and signalling. By HH26/27 FGF signalling is not detected and the notochord ends abruptly encircled by Sox2 expressing cells. Cell death is widespread in the tailbud and is promoted by retinoid signalling.

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Implanting Beads into Stage Late Stage Tail Bud in Vivo

HH stage 20/21 embryos were accessed in ovo for bead grafts. AGX-21 beads were soaked in RAR and RXR antagonists LG100815 and LG101208 (1 mM) or DMSO for controls, and heparin-coated beads were used to present FGF4 (100 μg/ml) or control (bovine serum albumen) BSA. Vitamin A-deficient quails were a kind gift of Malcolm Maden (Kings College London). Explants of chick tissue were isolated as indicated in the text and cultured as described previously [51] with mouse FGFR8b (250 ng/ml) (R&D Systems) with heparin (0.1 ng/ml) and BSA (0.0001%). and FGFR inhibitor PD173074 (250 mM) [76] or MEK antagonist PD184352 (3 mM) [77] or DMSO control; 9-cis RA (10 μM or 1 μM, Sigma) or all-trans RA (10 μM, 1 μM, or 100 nM, Sigma) had similar effects and were used as indicated in figure legends to control DMSO, RA synthesis inhibitor Disulflram (1 or 10 μM), or RAR and RXR antagonists LG100815 and LG101208 (5 mM) (Ligand Pharmaceuticals), or DMSO control, as described previously [11].

Cell Death Assay

The ApopTag In Situ Apoptosis Detection Kit (Chemicon) or NucViewTM 488 Caspase-3 substrate, a novel, fixable cell membrane-permeable fluorogenic caspase substrate that detects Caspase-3 activity within live cells (Biotium, 30029), was used according to the manufacturers’ instructions.

Cloning

A chick Crabp2 probe was generated from ChEST74f2 (BBRSC Chicken EST database). ChEST74f2 was sequenced and coding region (414 bp) and short flanking sequences identified. Blast searches indicated a single match to crabp2-like gene in meleagris gallopavo (accession number XM 003213787.1).

Supporting Information

Figure S1 Sox2 expression at HH20–21. Sox2 transcripts are detected transiently in the chick distal tail gut and are lost at HH20/21, after which this structure disappears and Sox2 is only detected in the neuroepithelium and at HH24 spreads into the position of the mesoderm progenitor domain (see Figure 4).

Figure S2 Expression of RARβ in the chick tailbud. In situ hybridisation for RARβ from middle to end of body axis elongation in chick (A–D). Low-level RARβ is detected in the chick tailbud. In all figures, stages are labelled; top rows are lateral views, bottom rows dorsal views. (A′) and (C′) are sagittal sections at the indicated stages. CNH, red dashed line; mesoderm progenitors, yellow dashed line; nt, neural tube; nc, notochord. Scale bars are 100 μm.

Figure S3 Crabp2 expression and regulation by retinoid signalling in the tailbud. Crabp2 expression at HH10 (A) and HH11/16 (A′) is not detected in the tailbud. HH19–22 tailbud explants treated with DMSO-only control (B) or RA [all trans RA, 100 nM] (B′) in which Crabp2 expression is increased (n = 2/2). HH19–22 tailbud explants treated with (C) DMSO only or the RA-synthesis inhibitor Disulflram (C′) in which Crabp2 expression was not detected. Explanted tissues were processed using an InsituPro machine (Intavis). Normal expression patterns were observed in at least four embryos per stage, per gene. Tailbud cell populations were analysed in sections prepared following standard cryosectioning. Immunocytochemistry in human embryos at CS12-17 for Sox2 and Bra was carried out using a standard protocol for wax sections, using citrate buffer antigen retrieval. Primary antibodies were as follows: Goat anti-Sox2 Immune Systems Ltd (GT15098; batch 401196) (1:100); Rabbit anti-Brachyury (Santa Cruz) (SC-20109) (1:50); and for Chick sections, Rabbit anti-Sox2 (Millipore Ab 5603) (1:100) and Goat anti-Brachyury R&D Systems. Secondaries were all from Molecular Probes and used at 1:200; for Human tissue, Donkey anti-Goat A594 and Donkey anti-rabbit-A488; and for Chick tissue, Donkey anti-Goat A488 and Donkey anti-Rabbit A594. Labelled sections were imaged using a Delta-Vision widefield microscope.

In Situ Hybridisation and Immunocytochemistry

Standard methods for whole mount in situ hybridisation were used. Explanted tissues were processed using an InsituPro machine (Intavis). Normal expression patterns were observed in at least four embryos per stage, per gene. Tailbud cell populations were

0.5 mg/ml 9-cis RA or DMSO for controls, and heparin-coated beads were used to present FGF4 (100 μg/ml) or control (bovine serum albumen) BSA. Vitamin A-deficient quails were a kind gift of Malcolm Maden (Kings College London). Explants of chick tissue were isolated as indicated in the text and cultured as described previously [51] with mouse FGFR8b (250 ng/ml) (R&D Systems) with heparin (0.1 ng/ml) and BSA (0.0001%); and FGFR inhibitor PD173074 (250 mM) [76] or MEK antagonist PD184352 (3 mM) [77] or DMSO control; 9-cis RA (10 μM or 1 μM, Sigma) or all-trans RA (10 μM, 1 μM, or 100 nM, Sigma) had similar effects and were used as indicated in figure legends to control DMSO, RA synthesis inhibitor Disulflram (1 or 10 μM), or RAR and RXR antagonists LG100815 and LG101208 (5 mM) (Ligand Pharmaceuticals), or DMSO control, as described previously [11].
is reduced (n = 5/5). Beads delivering control DMSO (D) or RAR/ RXR antagonists (D') were grafted into the HH20/21 tailbud and cultured for 24 h. RAR/RXR antagonists reduced Cited2 expression (n = 6/6), compared to DMSO controls (1/6). RAR antagonist beads placed in the flank attenuate RARb and Crabp2 expression. Scale bars, 100 µm, * bead position. (TIF)

**Figure S4** RAR antagonist alone elicits ectopic Bga expression. Schematic of bead grafting experiment (A), DMSO (B), and RAR antagonist delivering beads (B'). Ectopic Bga was detected in response to RAR antagonist (n = 5/12) but not DMSO beads (n = 0/8). Scale bar, 100 µm, * bead position. (TIF)

**Figure S5** RA increases cell death in tailbud explants. (A) HH19–22 tailbud explant pairs were cultured either in DMSO (B) or RA (B') in cell death was less pronounced; 3/5 explant pairs cultured in 1 µM atRA and 2/5 pairs at 0.1 µM atRA (unpublished data). (TIF)

**Figure S6** FGF regulation of apoptosis in the tailbud. (A, A') Exposure of HH19–22 tailbud explants to FGF8b (200 ng/ml) for 24 h reduced incidence of cell death as indicated by Caspase-3 activity (NucView in comparison with no growth factor control conditions (n = 4/4 explant pairs). (B, B') Exposure of HH19–22 tailbud explants to FGFR inhibitor PD173074 did not consistently alter levels of apoptosis as indicated by Caspase-3 activity, in comparison with control DMSO-only-treated tailbuds (PD173074, n = 6; DMSO, n = 4). Explants for FGF8 were imaged on a conventional wide-field microscope, and PD173074 experiments were imaged using confocal microscopy. (TIF)

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**Author Contributions**

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: KGS IO-M HH PAH. Performed the experiments: KGS IO-M HH PAH. Analyzed the data: KGS IO-M HH PAH. Wrote the paper: KGS IO-M HH.

**References**

1. Catala M, Teillet MA, Le-Douarin NM (1995) Organization and development of the tail bud analyzed with the quail-chick chimera system. Mech Dev 51: 51–63.
2. Wilson V, Olivera-Martinez I, Storey KG (2009) Stem cells, signals and vertebrate body axis extension. Development 136: 1591–1604.
3. Tzouanacou E, Wegener A, Wyneersch EJ, Wilson V, Nicolas JF (2009) Redefining the progression of lineage segregations during mammalian embryogenesis by clonal analysis. Dev Cell 17: 365–376.
4. Cambray N, Wilson V (2007) Two distinct sources for a population of maturing axial progenitors. Development 134: 2829–2840.
5. McGrew MJ, Sherman A, Lilloo SG, Ellard RM, Radcliffe PA, et al. (2008) Localized axial progenitor cell populations in the avian tail bud are not committed to a posterior Hox identity. Development 135: 2289–2299.
6. Murakami U, Kameyama Y (1985) Malformations of the mouse fetus caused by hypervitaminosis-A of the mother during pregnancy. Arch Environ Health 10: 727–741.
7. Kessel M, Gruss P (1991) Homoeotopic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. Cell 67: 89–104.
8. Shum AS, Poon LL, Tang WW, Koide T, Chan BW, et al. (1999) Retinoic acid induces down-regulation of Wnt-3a, apoptosis and diversion of tail bud cells to a neural fate in the mouse embryo. Dev Growth Differ 41: 17–30.
9. Takada S, Stark K, Shea M, Vassileva G, McMahon J, et al. (1994) Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. and Development 8: 174–189.
10. Aulehla A, Witte C, Brand-Saberi B, Kemsler R, Gosler A, et al. (2003) Wnt3a plays a major role in the segmentation clock controlling somitogenesis. Dev Cell 4: 395–406.
11. Dize del Corral R, Olivera-Martinez I, Goriel A, Gale E, Maden M, et al. (2003) Opposing FGF and retinoid pathways control ventral neural pattern, neuronal differentiation, and segmentation during body axis extension. Neuron 40: 63–79.
12. Olivera-Martinez I, Storey KG (2007) Wnt signals provide a timing mechanism for the FGF-retinoid differentiation switch during vertebrate body axis extension. Development 134: 2125–2135.
13. Wahl MB, Deng C, Lewandoski M, Pourquie O (2007) FGF signalling acts upstream of the NOCTH and WNT signaling pathways to control segmentation clock oscillations in mouse somitogenesis. Development 134: 4033–4041.
14. Streavids MP, Collin BJ, Storey KG (2010) Retinoic acid oscillators orchestrate fibroblast growth factor signalling to drive embryonic stem cell differentiation. Development 137: 801–809.
15. Sanders EJ, Khare MK, Ooi VC, Bellairs R (1986) An experimental and morphological analysis of the tail bud mesenchyme of the chick embryo. Anat Embryol (Berl) 174: 179–183.
16. Gomez C, Ozbudak EM, Wunderlich J, Baumann D, Lewis J, et al. (2008) Control of segment number in vertebrate embryos. Nature 454: 335–339.
17. Tenin G, Wright D, Ferjentsik Z, Bone R, McGrew MJ, et al. (2010) The chick somitogenesis oscillator is arrested before all paraxial mesoderm is segmented into somites. BMC Dev Biol 10: 24.
18. Young T, Rowland JE, van de Ven C, Bielacka M, Novoa A, et al. (2009) Cdx and Hox genes differentially regulate posterior axial growth in mammalian embryos. Dev Growth Differ 51: 516–526.
19. Savory JG, Bouchard N, Pierre Y, Rijii FM, De Repentigny Y, et al. (2009) Cdx2 regulation of posterior development through non-Hox targets. Development 136: 4099–4110.
20. Jacobs HV, Pownall ME, Slack JM (1998) Regulation of Hox gene expression and posterior development by the Xenopus caudal homologue Xcad3. Embryo J 17: 3413–3427.
21. Bel-Viard S, Issaki N, Krumlauf R (2002) Initiating Hox gene expression in the early chick neural tube: differential sensitivity to FGF and RA signaling subdivides the HoxB genes in two distinct groups. Development 129: 5103–5113.
22. Naiche LA, Holder N, Lewandoski M (2011) FGF4 and FGF8 comprise the wavefront activity that controls somitogenesis. Proc Natl Acad Sci U S A 108: 4018–4023.
23. Dobrovolskaia-Zavadskaja N (1927) Sur la mortification spontanee de la queue chez la souris nouveau-ne et sur l'existence d'un caractere hereditaire "non viable". C R Hebd Soc Biol 97: 114–116.
24. Herrmann BG, Labeit S, Pouska A, King TR, Lehrbach H (1998) Cloning of the T gene required in mesoderm formation in the mouse. Nature 343: 617–622.
25. Martin BL, Kimmel DB (2010) Brachyury establishes the embryonic mesodermal progenitor niche. Genes & Development 24: 2778–2783.
26. Reipujos S, Blencic A, Gale E, Maden M (2003) The control of morphogen signalling: regulation of the synclinium and cadherinism of retinoic acid in the developing embryo. Dev Biol 265: 224–237.
27. Yamaguchi TP, Takada S, Yoshikawa Y, Wu N, McMahon AP (1999) T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. Genes Dev 13: 3183–3190.
28. Harvey SA, Tumpel S, Dubrulle J, Schier AF, Smith JC (2010) No tail integrates two modes of mesoderm induction. Development 137: 1127–1135.
29. Abu-Abed S, Dolle P, Metzger D, Beckett B, 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 Dev 15: 226–240.
30. Ciruna BG, Schwartz L, Harpal K, Yamaguchi TP, Rossant J (1997) Chimeric analysis of fibroblast growth factor receptor-1 (Fgfr1) function: a role for Fgfr1 in morphogenetic movement through the primitive streak. Development 124: 2829–2841.
31. Sakai Y, Meno C, Fujii H, Nishino J, Shiraishi H, et al. (2001) The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the antero-posterior axis within the mouse embryo. Genes Dev 15: 2125–2135.
32. Deng C, Bedford M, Li G, Xu X, Yang X, et al. (1997) Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. Dev Biol 185: 42–54.
33. Ribes V, Le Roux I, Rhinn M, Schuhbauer B, Dolle P (2009) Early mouse embryonic relies on crosstalk between retinoic acid, Shh and Fgf signalling pathways. Development 136: 665–676.
34. Martin BL, Kimelman D (2012) Canonical Wnt signaling dynamically controls multiple stem cell fate decisions during vertebrate body formation. Developmental Cell 22: 223–232.
35. Cunningham TJ, Zhao X, Duister G (2011) Uncoupling of retinoic acid signaling from tailbud development prior to termination of body axis extension. Genesis.
36. Griley BB (1969) Analysis of the embryonic sources and mechanisms of development of the posterior levels of chick neural tubes. J Morph 126: 463–502.
37. Minowada G, Jarvis LA, Chi CL, Neubuser A, Sun X, et al. (1999) Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. Development 126: 4465–4475.
38. Miller SA, Briglin A (1996) Apoptosis removes chick embryo tail gut and remnant of the H-otic streak. Developmental Dynamics 206: 212–218.
39. O’Rahilly R, Muller F (2003) Somites, spinal Ganglia, and centra. Enumeration and interrelationships in staged human embryos, and implications for neural tube defects. Cells, Tissues, Organs 173: 75–92.
40. Takemoto T, Uchikawa M, Yoshida M, Bell DM, Lovell-Badge R, et al. (2011) Thbx-dependent Sox2 regulation determines neural or mesodermal fate in axial stem cells. Nature 470: 394–398.
41. Uchikawa M, Ishida Y, Takamoto T, Kamachi Y, Kondoh H (2003) Functional analysis of chicken Sox enhancers highlights an array of diverse regulatory elements that are conserved in mammals. Dev Cell 4: 509–519.
42. Takemoto T, Uchikawa M, Kamachi Y, Kondoh H (2006) Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1. Development 133: 297–306.
43. de la H, Vicancio-Ruiz MM, Tiemann P, Stunnenberg H, Dejene A (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. Nature 343: 177–180.
44. Rossant J, Zunghil K, Cado D, Shago M, Giguere V (1991) Expression of a retinoic acid response element-lacZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. Genes Dev 5: 1333–1344.
45. Bastien J, Rochette-Egly C (2004) Nuclear retinoid receptors and the transcription of retinoid-target genes. Gene 320: 1–16.
46. Durand B, Saunders M, Leroy P, Leid M, Chambon P (1992) All-trans and 9-cis retinoic acid induction of CRABPII transcription is mediated by RAR-RXR heterodimers bound to DR1 and DR2 repeated motifs. Cell 71: 73–85.
47. Kitamoto T, Momoi M, Momoi T (1989) Expression of cellular retinoic acid-binding protein II (chick-CRABP II) in the chick embryo. Biochem Biophys Res Commun 164: 531–536.
48. Maden M (1994) Distribution of cellular retinoic acid-binding proteins I and II in the chick embryo and their relationship to teratogenesis. Teratology 50: 294–304.
49. Perez-Castro AV, Toth-Roger LE, Wei LN, Nguyen-Huu MC (1989) Spatial and temporal pattern of expression of the cellular retinoic acid-binding protein and the cellular retinol-binding protein during mouse embryogenesis. Proc Natl Acad Sci U S A 86: 8813–8817.
50. Ruberte E, Friederich V, Morris-Kay G, Chambon P (1992) Differential distribution patterns of CRABP I and CRABP II transcripts during mouse embryogenesis. Development 115: 973–987.
51. Diez del Corral R, Breitkreuz DN, Storey KG (2002) Onset of neuronal differentiation is regulated by paraxial mesoderm and requires attenuation of FGF signalling. Development 129: 1681–1691.
52. Kostetskii I, Yuan SY, Kostetskaya E, Linak KK, Blanchet S, et al. (1990) Initial retinoid requirement for early axis development coincides with retinoid receptor coexpression in the precardiac fields and induction of normal cardiovascular development. Dev Dyn 213: 188–198.
53. Niederreither K, Subbarayan V, Dolle P, Chambon P (1999) Embryonic retinoic acid synthase is essential for early mouse post-implantation development. Nat Genet 21: 444–448.
54. Bellairs R (1961) Cell death in chick embryos as studied by electron microscopy. J Anat 95: 54–60.
55. Mills CL, Bellairs R (1989) Mitosis and cell death in the tail of the chick embryo. Anat Embryol 180: 301–308.
56. Sureka D, Hudgins L (2010) Fibrolast growth factor receptor 2 and its role in caudal appendage and craniosynostosis. J Craniofac Surg 21: 1346–1349.
57. Gaunt SJ, Drage D, Trubshaw RC (2008) Increased CdX protein dose effects upon axial patterning in transgenic lines of mice. Development 135: 2511–2520.
58. Isaacs HV, Pownell ME, Slack JM (1994) FGF regulates Xbra expression during Xenopus gastrulation. EMBO J 13: 4469–4481.
59. Arnold SJ, Stappert J, Bauer A, Kispert A, Herrmann BG, et al. (2000) Brachyury is a target gene of the Wnt/beta-catenin signaling pathway. Mech Dev 91: 249–258.
60. Martin BL, Kimelman D (2008) Regulation of canonical Wnt signaling by Brachyury is essential for posterior mesoderm development. Development Cell 15: 121–133.
61. Hofmann M, Schuster-Gosler K, Watabe-Rudolph M, Aulehla A, Herrmann BG, et al. (2004) WNT signaling, in synergy with T/T/BXN6, controls Notch signaling by regulating DLL1 expression in the presomitic mesoderm of mouse embryos. Genes Dev 18: 2712–2717.
62. Wardle FC, Papasanoou VE (2008) Treating T-box targets in early mesoderm. Curr Opin Genet Dev 18: 418–425.
63. Abu-abled S, Dolle P, Metzger D, Wood C, MacLean G, et al. (2003) Developing with lethal RA levels: genetic ablation of Rar can restore the viability of mice lacking Cyp26a1. Development 130: 1449–1459.
64. Iulianella A, Beckett B, Pelkovich M, Lohnes D (1999) A molecular basis for retinoic acid-induced axial truncation. Dev Biol 205: 33–46.
65. Bromlai V, Klimka T, Egly JM, Hanny F (2002) Promoters of FGFs reveal a unique regulation by unliganded RARalpha. J Mol Biol 319: 715–728.
66. Zhao X, Sirbu IO, Mic FA, Molokova N, Molokov A, et al. (2009) Retinoic acid promotes limb induction through effects on body axis extension but is unnecessary for limb patterning. Curr Biol 19: 1050–1057.
67. Astrom A, Tavakkol A, Pettersson U, Cromie M, Elder JT, et al. (1991) Molecular cloning of two human cellular retinoic acid-binding proteins (CRABP). Retinoic acid-induced expression of CRABP-II but not CRABP-I in adult human skin in vivo and in skin fibroblasts in vitro. The Journal of Biological Chemistry 266: 17662–17666.
68. Giguere V, Lyn S, Yip P, Sun CH, Amin S (1990) Molecular cloning of cDNA encoding a second cellular retinoic acid-binding protein. Proc Natl Acad Sci U S A 87: 6233–6237.
69. Chen AC, Gudas LJ (1996) An analysis of retinoic acid-induced gene expression and metabolism in AB1 embryonic stem cells. J Biol Chem 271: 14971–14980.
70. Sonneveld E, van den Brink CE, van der Leede BJ, Maden M, van der Saag PT (1999) Embryonal carcinoma cell lines stably transfected with mRARbeta2-lacZ: sensitive system for measuring levels of active retinoids. Exp Cell Res 250: 204–297.
71. Schoenwolf GC (1981) Morphogenetic processes involved in the remodeling of the tail region of the chick embryo. Anat Embryol Berl 173: 183–207.
72. Tickle G, Craswely A, Farrar J (1989) Retinoic acid application to chick wing buds leads to a dose-dependent reorganization of the apical ectodermal ridge that is mediated by the mesenchyme. Development 106: 691–705.
73. Tickle G, Craswely A, Farrar J (1989) Retinoic acid application to chick wing buds leads to a dose-dependent reorganization of the apical ectodermal ridge that is mediated by the mesenchyme. Development 106: 691–705.
74. Hamburger H, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J Exp Morphol 9: 49–92.
75. Chapman SC, Collignon J, Schoenwolf GC, Laumens A (2001) Improved method for chick whole-embryo culture using a filter paper carrier. Dev Dyn 220: 284–289.
76. Mohammadi M, McMahon G, Sun L, Tang C, Hirth P, et al. (1997) Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. Science 276: 955–960.
77. Davies SP, Reddy H, Gavino M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95–105.
78. Brown JM, Storey KG (2000) A region of the vertebrate neural plate in which neighbouring cells can adopt neural or epidermal cell fates. Curr Biol 10: 869–872.