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Retinoic acid signaling regulates embryonic clock *hairy2* gene expression in the developing chick limb

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ABSTRACT

Embryo development proceeds under strict temporal control and an embryonic molecular clock (EC), evidenced by cyclic gene expression, is operating during somite formation and limb development, providing temporal information to precursor cells. In somite precursor cells, EC gene expression and periodicity depends on Retinoic acid (RA) signaling and this morphogen is also essential for limb initiation, outgrowth and patterning. Since the limb EC gene *hairy2* is differentially expressed along the proximal–distal axis as growth proceeds, concomitant with changes in flank-derived RA activity in the mesenchyme, we have interrogated the role of RA signaling on limb *hairy2* expression regulation. We describe RA as a positive regulator of limb *hairy2* expression. Ectopic supplementation of RA induced *hairy2* in a short time period, with simultaneous transient activation of Erk/MAPK, Akt/PI3K and Gli3 intracellular pathways. We further found that FGF8, an inducer of Erk/MAPK, Akt/PI3K pathways, was not sufficient for ectopic *hairy2* induction. However, joint treatment with both RA and FGF8 induced *hairy2*, indicating that RA is creating a permissive condition for p-Erk/p-Akt action on *hairy2*, most likely by enhancing Gli3-A/Gli3-R levels. Finally, we observed an inhibitory action of BMP4 on *hairy2* and propose a model whereby RA shapes limb *hairy2* expression during limb development, by activating its expression and counteracting the inhibitory action of BMP4 on *hairy2*. Overall, our work reports a novel role for RA in the regulation of limb clock *hairy2* gene expression and elucidates the temporal response of multiple intracellular pathways to RA signaling in limb development.

1. Introduction

Spatiotemporal fine-tuned gene expression regulation is a fundamental trait in all biological processes, including embryogenesis. An embryo time-counting mechanism was first reported by describing cyclic expression of *hairy1*, a Hairy/Enhance-of-split (HES) gene, underlying the periodicity of somite formation [1]. Multiple genes belonging to this embryonic molecular clock (EC) have been reported in different species and belonging to the Notch, FGF and WNT signaling pathways [2]. A decade after the somitogenesis clocks discovery, a similar molecular clock was found to operate in the chick distal limb mesenchyme [3]. This tissue presents cyclic expression of another HES family member, *hairy2*, in the chondrogenic precursor cells with a 6 h periodicity [3]. In the following years, oscillations of *HES* expression have also been described in human mesenchymal stem cells [4], mouse neural progenitor cells [5] and in embryonic stem cells [6]. Thus, gene oscillations are a wide-spread mechanism, providing temporal information to multiple systems.

EC genes are regulated by retinoic acid (RA) signaling in the presomitic mesoderm (PSM) [7–9]. Retinoic acid (RA) is a morphogen, derived from vitamin A, which regulates gene expression through its interaction with nuclear receptors [10]. During early embryonic phases, the RA synthesizing enzyme *raldh2* is expressed in the PSM overlapping the *fgf8* expression domain [8]. Over time, the antagonistic action of RA and FGF8 signaling generates opposing anterior-*raldh2* and posterior-*fgf8* gradients, which are absolutely required for proper somitogenesis [7,8,11]. We have further described that RA ensures timely somite formation through modulation of Gli activity [12]. In fact, SHH signaling deprivation in chick PSM delayed both EC periodicity and somite formation rate, which was rescued by RA-mediated Gli activity modulation [12], suggesting the ability of RA to functionally replace SHH signaling.

The developing limb begins as a small bud of homogenous mesenchymal cells under the influence of the opposing proximal–distal RA signaling and the distal–proximal FGF signaling deriving

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from the apical ectodermal ridge (AER) [13]. These two morphogen gradients are essential for limb proximal–distal (PD) outgrowth and patterning [14]. RA also participates in limb anterior–posterior (AP) patterning, since it activates *shh* expression in the zone of polarizing activity (ZPA) [15,16]. While AER-FGFs signal through Erk/MAPK and Akt/PI3K pathway activation [17–19], Gli1–3 proteins function as the intracellular effectors of ZPA-SHH activity [20,21]. As the limb develops, the RA domain is proximalized due to the polarized gene expression distribution of its synthesizing (*Raldh2*) and degrading (*Cyp26*) enzymes, in the embryo flank and distal limb mesenchyme, respectively [22–24]. Importantly, the limb EC *hairy2* gene presents distinct domains of expression during limb development [3], concomitant with the changing influence of RA and FGF signaling in the limb field. In the present study, we have interrogated the role of RA signaling on the regulation of limb EC *hairy2* gene expression. We further assessed the temporal response of the chick embryo forelimb tissue to RA by elucidating the signaling pathways which this morphogen modulates over time.

2. Materials and methods

2.1. Eggs and embryos

Fertilized Gallus gallus eggs were incubated at 37.8 °C in a 49% humidified atmosphere and staged according to Hamburger and Hamilton (HH) classification [25]. All the experiments were performed in stage HH22–24 forelimb buds.

2.2. Microsurgical ablation of ZPA tissue

A window was cut in the shell of incubated eggs and the vitelline membrane was carefully removed. The ZPA was microsurgically ablated from the right wing bud of embryos using a tungsten needle. As a control, ZPA extirpated embryos were randomly selected for direct fixation and hybridization with *shh*. Operated embryos were re-incubated for 6–7 h, either collected in PBS and fixed for in situ hybridization or subjected to additional manipulations.

2.3. Bead implantation experiments

AG1-X2 beads (Bio-Rad) were soaked for 20 min at room temperature in *all-trans* RA (5 μg/μl; Sigma) in DMSO. The beads were implanted in ovo into the mesoderm of right chick wing buds at the desired position and for the desired time. Beads soaked in DMSO served as control and they did not show any effect on gene expression.

2.4. In situ hybridization and imaging

In situ hybridization was performed as previously described [26], using antisense digoxigenin-labeled RNA probes: *shh* [27] and *hairy2* [28]. Limbs processed for in situ hybridization were photographed using an Olympus DP71 digital camera coupled to an Olympus SZX16 stereomicroscope.

2.5. Immunoblot analysis

RA-beads were implanted in the forelimb AND and incubated either for 1 h or 6 h time period. Experimental and contralateral control limbs were surgically ablated and divided along the proximal–distal axis and the untreated halves were discarded. Portions from at least twelve different limbs were collected and total protein was extracted from each limb pool. 10 μg and 50 μg of protein extracts were loaded per well on a 12% and 7% SDS–PAGE minigel, respectively, for Erk/MAPK, Akt/PI3K and Gli3 western-blots. Blots were probed with p44/42 MAPK, phosphor-p44/42 MAPK, Akt, phosphor-Akt (Cell signaling) and Gli3 polyclonal [21] primary antibodies. β-tubulin (Abcam) antibody was used as loading control. Blots were incubated with anti-rabbit secondary antibody (Abcam), developed with Super Signal West Femto Substrate (Pierce Biotechnology, Inc., Rockford, IL) and exposed in Chemidoc (Bio-Rad). Bands were quantified using Quantity one (Bio-Rad) and normalized with loading control.

3. Results and discussion

The chick distal limb mesenchyme displays distinct *hairy2* expression patterns between stages HH20–28, cyclically recapitulated in the sub-ridge mesenchyme (DCD, distal cyclic domain) every 6 h [3,29]. *hairy2* transcripts are always detectable in the central mesenchymal limb domain containing the muscle precursors (CPD, central positive domain) and in the posterior limb encompassing the ZPA (PPD, posterior positive domain) (Fig. 1A). In contrast, *hairy2* expression is absent in both the anterior and posterior limb mesenchyme (AND, anterior negative domain; PND, posterior negative domain) (Fig. 1A). Understanding the regulatory mechanisms of limb *hairy2* expression is very important since it is proposed to provide temporal information to chondrogenic precursor cells before they enter their differentiation program [3]. Taking into account the previous knowledge of RA regulatory effects on EC gene expression during somitogenesis

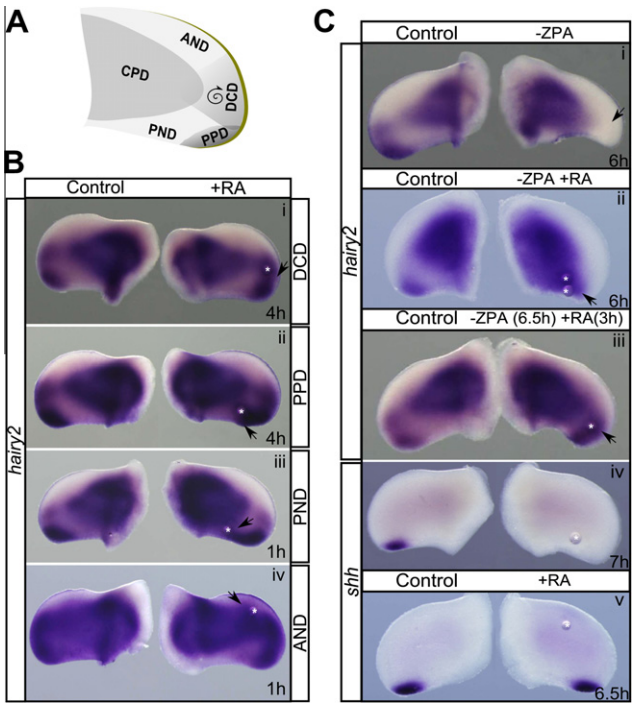

Fig. 1. RA signaling induces limb *hairy2* expression in a SHH-independent manner. (A) Schematic representation of *hairy2* expression domains in HH24 chick forelimb. *hairy2* is always observed in the central positive domain (CPD) and in the posterior positive domain (PPD). *hairy2* is absent from both anterior negative (AND) and posterior negative (PND) domains and is cyclically expressed in the distal cyclic domain (DCD), with a 6 h periodicity. (B) *hairy2* is upregulated by implantation of RA-beads in all limb domains. (C) Distal *hairy2* expression is lost upon ZPA ablation (i) and rescued by RA-bead implantation, either immediately (ii) or after 6.5 h of tissue manipulation (iii). RA-induced *hairy2* expression is not mediated by *shh* induction (iv, v). Dorsal view; anterior to the top. *RA-Beads. Arrows indicate altered *hairy2* expression.

and the crucial role of RA in limb development, we have assessed how RA signaling impacts limb EC *hairy2* gene expression.

3.1. RA signaling induces limb hairy2 expression

In order to assess the role of RA signaling on limb *hairy2* expression, RA-soaked beads were implanted in different distal limb domains (Fig. 1A and B). *hairy2* was upregulated in both positive domains, DCD and PPD (Fig. 1Bi, ii, $n = 14/14$; $n = 13/13$, respectively), and ectopically induced in both native *hairy2*-negative domains, PND and AND (Fig. 1Biii, iv, $n = 51/51$; $n = 29/34$, respectively). The latter was true as soon as in 1 h of incubation (PND: $n = 14/14$; AND: $n = 6/7$) revealing a short-term effect of RA on *hairy2*. It is known that RA can induce *shh* expression in the anterior limb mesenchyme [27], so we assessed if the effect of RA on *hairy2* expression is mediated through *shh*. With this purpose, microsurgical ablation of the ZPA (SHH source) was performed in the experimental limb, leaving the control unhampered. Upon 6 h of ZPA ablation, *hairy2* expression is abolished in the distal limb (Fig. 1Ci). RA-bead implantation in the distal limb either immediately after, or following 6 h of ZPA ablation, was still capable of inducing *hairy2* expression (Fig. 1Cii, iii). This effect was not through *shh*, since within this time-frame *shh* expression remained absent (Fig. 1Civ). RA-mediated induction of *hairy2* expression in the AND also occurred independently of ectopic *shh* induction (Fig. 1Cv).

These results clearly indicate RA as a positive regulator of limb *hairy2* expression. This is in accordance with *hairy2* mouse homolog – *hes1* – upregulation obtained upon retinol treatment of mouse limb cultures [30]. We further show that RA-mediated induction of *hairy2* is not through SHH and that it occurs in a short-term manner.

3.2. RA modulates Gli3 activity in the anterior limb, creating a permissive condition for hairy2 expression

RA is capable of inducing ectopic *hairy2* in the AND (Fig. 1Biv), a high Gli3-R activity region [21]. RA is reported to modulate Gli activity [31] and our lab has previously proposed this mechanism in the regulation of *hairy2* expression in the PSM [12], prompting us to test whether RA-induction of *hairy2* in the anterior limb could also involve Gli3 activity modulation. We found that RA increased relative Gli3 activity levels by 17% in the anterior limb as soon as in 1 h of incubation (Fig. 2). This short-term effect was transient, since after 6 h of incubation the Gli3-A/Gli3-R ratio was unaltered relative to the control. This observation further supports that RA-ectopic *hairy2* expression is not mediated by SHH signaling, since direct treatment of the anterior limb with SHH only altered Gli3 activity after a minimum of 4 h of incubation [32].

3.3. RA-mediated induction of hairy2 involves a short-term, transient increase in Erk phosphorylation levels

RA has also been reported to stimulate Erk phosphorylation in multiple systems, including the chick retina [33]. To unveil the signaling pathways involved in RA-mediated ectopic induction of *hairy2*, we implanted RA-soaked beads in the AND and evaluated Erk/MAPK signaling, since it underlies *hes1* oscillations in C3H10T1/2 cells [34] and *hes7* in mouse PSM [35,36]. This was performed using samples with both short (1 h) and long (6 h)-term incubations following RA-bead implantation in the AND: a *hairy2* negative, RA- and SHH-free tissue (Fig. 3A,B). Akt/PI3K pathway was assessed in the same samples. We observed a 77% elevation in p-Erk after 1 h of RA exposure (Fig. 3A,B). This increase in p-Erk levels was very rapid (1 h) and transient in nature, as it was not sustained upon longer incubation periods (Fig. 3A,B). In these

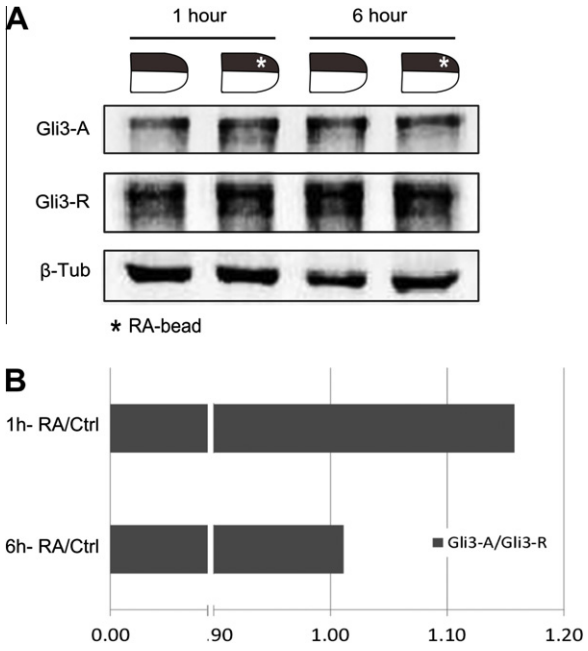

Fig. 2. RA establishes a permissive state for *hairy2* expression, mediated by a short-term, transient increase in Gli3-A/Gli3-R levels. (A) Immunoblots for Gli3-A, Gli3-R and β-tubulin (loading control) using total protein extracts from RA-treated anterior limb halves and their contralateral controls, as schematically represented, after 1 h and 6 h of incubation. (B) Fold change in the levels of Gli3-A/Gli3-R obtained in treated tissues relative to controls. RA increases Gli3-A/Gli3-R ratio in 1 h and this is not maintained after 6 h incubation. *RA-beads.

conditions, p-Akt levels were also slightly increased only after 1 h of incubation (Fig. 3A, B). In summary, we report that RA is capable of rapidly inducing Erk phosphorylation and p-Akt to a lesser extent, in the anterior distal limb. Although this is a transient effect, it is sufficient for *hairy2* induction, as previously shown (Fig. 1Biv).

Our data collectively show that RA induces Erk, Akt and Gli3 activity in the AND. Next, we interrogated whether an increase in p-Erk/p-Akt was sufficient for ectopic *hairy2* by implanting a bead imbibed in the Erk/MAPK and Akt/PI3K activator FGF8 [37]. *hairy2* was never induced in these conditions, even after 7 h of incubation (Fig. 3Ci). However, if we co-implanted RA- and FGF8-beads, *hairy2* was now upregulated around the FGF8 bead (Fig. 3Cii). This effect is lost if FGF8 is not present within the first 1 h of RA treatment (Fig. 3Ciii), strongly suggesting that RA creates a short-term, permissive condition for FGF8-mediated induction of *hairy2* expression in the AND, most probably through Gli3 activity modulation.

A possibility which cannot be excluded is that RA creates a permissive condition by relieving *hairy2* from an inhibitory signal operating in the AND. A good candidate is the Bone Morphogenetic Protein (BMP), since *bmp4* is expressed in the anterior limb mesenchyme [38] corresponding to the *hairy2* AND. Moreover, BMPs are known to inhibit Erk/MAPK signaling [39] and to increase Gli3-R levels [40], suggesting that Bmp4 might be a negative regulator of *hairy2*. To test this possibility, we implanted BMP4-soaked beads in the distal *hairy2* positive domain, which markedly down-regulated *hairy2* expression (Fig. 3Civ; $n = 6/7$). These results indicate that RA could be acting on *hairy2* expression in the AND by relieving BMP4 inhibitory signal. Supporting this hypothesis, RA-mediated repression of BMP4 activity has been reported [41,42].

3.4. Proposed role for RA signaling on limb hairy2 expression

Collectively, our work evidences RA as a positive regulator of limb *hairy2* expression, functioning simultaneously as a permissive

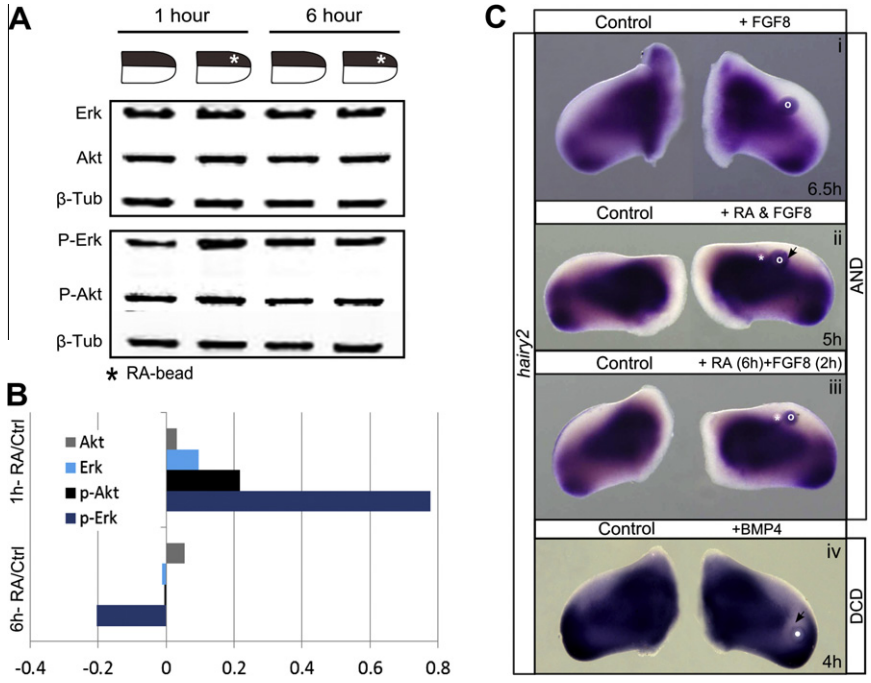

Fig. 3. Short-term, transient activation of Erk/MAPK and Akt/PI3K pathways underlies RA-induced *hairy2* expression. (A) Immunoblots for Erk, p-Erk, Akt, p-Akt and β-tubulin (loading control) using total protein extracts from anterior RA-bead implanted limb halves and their contralateral controls, as schematically represented, after 1 h and 6 h of incubation. (B) Fold change in Erk, p-Erk, Akt and p-Akt levels obtained in RA-treated tissues relative to control. RA increases both p-Erk and p-Akt levels in 1 h and this is not maintained after 6 h incubation. (C) In situ hybridization for *hairy2* revealing that FGF8 alone cannot induce ectopic expression (i), while co-implantation of FGF8- and RA-beads induces *hairy2* in the AND (ii). When the tissue is treated with RA for 6 h prior to FGF8-bead implantation, *hairy2* is no longer induced (iii), supporting a short-term effect of RA in creating a permissive state for FGF8-mediated *hairy2* induction. (iv) BMP4 is an inhibitor of *hairy2* expression. Dorsal view; anterior to the top. *RA-beads; ºFGF8-beads; •BMP4-beads. Arrows indicate altered *hairy2* expression.

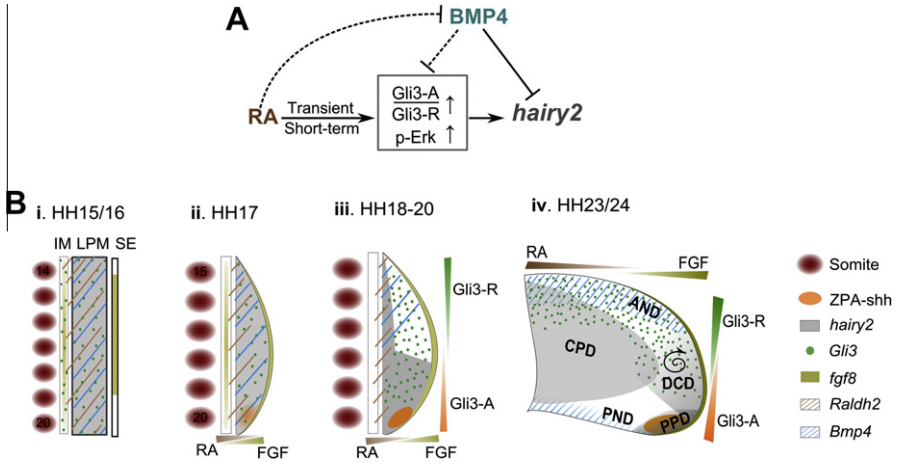

Fig. 4. Proposed model of how RA participates in shaping *hairy2* expression patterns during limb development. (A) RA rapidly induces a transient increase in both p-Erk and Gli3-A/Gli3-R levels, resulting in induction of *hairy2* expression. This effect may be through BMP4 signaling inhibition [41,42]. Moreover, we observe that BMP4 is an inhibitor of *hairy2* expression, hence it could be defining the limb *hairy2* AND. (B) In early wing bud development (i, ii), *raldh2, gli3* and *bmp4* are co-expressed throughout the entire tissue [22,38] [Geisha ID: 42Q]. In these conditions, RA is capable of inducing *hairy2* with concomitant inhibition of BMP4 [41,42] and Gli3 activity [31]. As the limb grows and counteracting Gli3-A and Gli3-R gradients are established by ZPA-SHH signaling, *hairy2* expression is differentially distributed along the limb AP axis (iii), restricted to the distal region where *bmp4* is absent [38]. Finally, when the opposing proximal–distal RA and FGF gradients no longer overlap (iv), *hairy2* presents distinct expression domains: constant expression in the proximal-most region, near the RA source (CPD), absence from the BMP4-rich regions (AND and PND) and oscillatory expression in the DCD [3], a tissue under joint AER/FGF and ZPA/SHH signaling.

and instructive signal through Gli3 modulation and Erk phosphorylation, respectively (Fig. 4A). RA promotes a simultaneous short-term, transient increase in p-Erk and Gli3A/Gli3R ratio in the AND, possibly by counteracting BMP4 [41,42]. Accordingly, we show that BMP4 is able to repress *hairy2* and suggest that it could be the signaling molecule defining the *hairy2* AND in the chick limb. Moreover, our data lead us to propose that RA has a role in defining the *hairy2* expression patterns during different phases of limb bud development, as schematized in Fig. 4B. The presumptive limb mesenchyme (stage HH15–HH17) co-expresses *raldh2, bmp4* and *hairy2* throughout the entire tissue [3,22,24,43,44]. High levels of RA in the presumptive wing bud [45] may enable *hairy2* expression by inhibiting both BMP4 and Gli3R activity. On the other hand, p-Erk levels are also expected to be high in these stages due to both

RA and AER-FGFs. Once *shh* expression is initiated at stage HH17 [46], opposing gradients of Gli-A and Gli-R will eventually be established in the limb mesenchyme [20,21,32] creating asymmetries in Gli3-A/Gli3-R levels along the limb AP axis. Moreover, SHH-mediated inhibition of *bmp4* expression impacts its AP distribution in the limb [47]. Accordingly, from stage HH18 onwards *hairy2* presents posterior positive and anterior negative limb expression domains (Fig. 4B) [3]. As the limb grows (stage HH23/24), counteracting FGF and RA gradients are displaced along the limb PD axis and the multiple *hairy2* expression domains are established (Figs. 1A and 4Biv), including the DCD, where cyclic gene expression occurs in the chondrogenic precursor cells [3].

This work provides novel insights on both EC gene expression regulation and RA-mediated control of signaling pathways during limb development, supporting RA functional relevance in limb PD outgrowth and patterning [48]. From our results and previous findings in the PSM [12,35], it is tempting to postulate that the limb signaling centers, AER (FGF source) and ZPA (SHH-producing tissue), are also involved in the establishment and regulation of EC *hairy2* gene expression in the developing limb. It is clear that a tight regulatory mechanism on limb *hairy2* gene expression is operating, similar to what occurs in the PSM underlying periodic somite formation, thus emphasizing the importance of the EC operating in the chick forelimb bud.

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