Joint interpretation of AER/FGF and ZPA/SHH over time and space underlies hairy2 expression in the chick limb

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Summary

Embryo development requires precise orchestration of cell proliferation and differentiation in both time and space. A molecular clock operating through gene expression oscillations was first described in the presomitic mesoderm (PSM) underlying periodic somite formation. Cycles of HES gene expression have been further identified in other progenitor cells, including the chick distal limb mesenchyme, embryonic neural progenitors and both mesenchymal and embryonic stem cells. In the limb, hairy2 is expressed in the distal mesenchyme, adjacent to the FGF source (AER) and along the ZPA-derived SHH gradient, the two major regulators of limb development. Here we report that hairy2 expression depends on joint AER/FGF and ZPA/SHH signaling. FGF plays an instructive role on hairy2, mediated by Erk and Akt pathway activation, while SHH acts by creating a permissive state defined by Gli3-A/Gli3-R>1. Moreover, we show that AER/FGF and ZPA/SHH present distinct temporal and spatial signaling properties in the distal limb mesenchyme: SHH acts at a long-term, long-range on hairy2, while FGF has a short-term, short-range action. Our work establishes limb hairy2 expression as an output of integrated FGF and SHH signaling in time and space, providing novel clues for understanding the regulatory mechanisms underlying HES oscillations in multiple systems, including embryonic stem cell pluripotency.

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Introduction

Periodic events underlie many aspects of our lives and some cell molecular oscillators have already been characterized and revealed to be crucial in insuring that the genetic/biochemical/morphological processes occur in their optimal temporal niche. One of such oscillators is the embryonic HES (Hairy/Enhancer of split)-based molecular clock (Pourquié, 2011) which was firstly identified by describing cyclic expression of avian hairy1/2 genes during timely somite formation (Palmeirim et al., 1997; Jouve et al., 2000). Since then, this molecular oscillator has been described in multiple animal models (Andrade et al., 2007; Krol et al., 2011) and in a great variety of cells, including limb chondrogenic precursor cells (Pascoal et al., 2007; Aulehla and Pourquié, 2008), embryonic neural progenitors (Shimjo et al., 2008) and both mesenchymal (William et al., 2007) and embryonic stem cells (Kobayashi et al., 2009). Furthermore, major signaling pathways such as Notch, Wnt and FGF have been implicated in the molecular clock machinery (Dequèant et al., 2006; Krol et al., 2011; Pourquié, 2011). Mutations in genes associated with this oscillator underlie vertebral human malformations (Andrade et al., 2007; Pourquié, 2011; Sparrow et al., 2012), revealing that a tight regulation of HES biological activity is crucial for normal embryogenesis. Interestingly, many of these congenital anomalies also include limb defects (Andrade et al., 2007; Turnpenny et al., 2007; Sparrow et al., 2012).

The developing limb begins as a small bud of homogenous mesenchymal cells and gets sculptured into a 3D adult limb through coordinated growth and patterning. FGFs produced by the distal apical ectodermal ridge (AER) dictate proximal–distal (PD) outgrowth and maintain the distal limb mesenchyme cells in an undifferentiated, proliferative state, mediated by Erk/MAPK and Akt/P13K pathways (Kawakami et al., 2003; Corson et al., 2003; Towers and Tickle, 2009). SHH produced by the zone of polarizing activity (ZPA) located at the posterior distal margin of the limb bud, governs anterior–posterior (AP) patterning (Riddle et al., 1993; Towers and Tickle, 2009). This is mediated through a SHH-dependent posterior-to-anterior gradient of Gli activator levels and an opposing Gli repressor gradient (Wang et al., 2000; Ahn and Joyner, 2004). In the chick distal-most limb mesenchyme, hairy2 is cyclically expressed with a 6 hour periodicity (Pascoal et al., 2007; Aulehla and Pourquié, 2008), whereas it is permanently expressed in the posterior limb region...
hairy2 mirrors FGF/SHH signaling

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The expression of hairy2 is an instructive signal that interacts with FGF and SHH signaling pathways in chick limb patterning. hairy2 expression is induced by FGF signaling, which is mediated by Erk and Akt pathways. In turn, SHH signaling creates a permissive state for FGF pathway activation.

In this study, the authors investigated the role of hairy2 expression in chick limb patterning. They demonstrated that hairy2 expression is an output of both FGF and SHH activities, which create a temporally and spatially regulated mechanism involving chick limb progenitor cells.

The results provide insights into the integrated signaling mechanisms that control limb development, highlighting the importance of hairy2 in instructive signaling.

**Materials and Methods**

**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 classification. All experiments were performed in stage HH22-24 forelimb buds.

**Microsurgical ablation of AER and ZPA tissue**

A window was cut in the shell of incubated eggs, and the vitelline membrane was removed carefully. AER or ZPA was microsurgically ablated from the right wing bud of embryos using a tungsten needle. As a control, AER or ZPA explants were used. operated embryos were re-incubated for different time periods (15 minutes to 22 hours), collected in PBS and fixed for in situ hybridization.

**TUNEL assay**

Apoptosis was analyzed using the Cell Death Detection Kit (Roche) in limb sections with and without AER or ZPA, after 2 and 6 hours of incubation, respectively. AER tissue was fixed overnight in 4% paraformaldehyde (PFA) in PBS, dehydrated, imbibed in paraffin and sliced into 6 μm sections. Sections were rehydrated, permeabilized with Proteinase K treatment for 7.5 minutes at room temperature, and washed in PBS. Positive control embryos were stained with DNease at 37°C for 30 minutes. Sections were incubated for 2–4 hours at 37°C with the TUNEL solution mix and washed at least three times in PBS before visualization.

**Cell graft experiments**

Clumps of QT6 quail fibroblasts stably transfected with an empty vector or with a construct carrying the SHH-coding region (QT6-SHH) (Duprez et al., 1998) were grafted in the posterior margin of the limb mesenchyme following ZPA ablation. Grafted embryos were re-incubated for 4–8 hours and processed for in situ hybridization.

**Bead implantation experiments**

Heparin or agarose beads (Sigma, S2526) and Affigel blue beads (Bio-Rad) were soaked for 1 hour at room temperature in recombinant human FGF8 or FGF2 (1 μg/μl; R&D Systems) and SHH (4 μg/μl; R&D Systems) proteins in PBS, respectively. The beads were implanted in vivo into the mesoderm of chick wing buds and beads soaked in PBS served as control. AG1-X2 beads (Bio-Rad) were incubated in 1% SDS for 1 hour at room temperature and washed in PBS.

**In situ hybridization and imaging**

In situ hybridization was performed as previously described (Henriques et al., 1995), using antisense digoxigenin-labelled RNA probes: shh (Riddle et al., 1993), hairy2 (Jouve et al., 2000), fgf8 (Crossley et al., 1996) and patched (Marigo and Tabin, 1996). Limbs processed for in situ hybridization were photographed using an Olympus DP71 digital camera coupled to an Olympus SZX16 stereomicroscope.

**Immunoblot analysis**

Western blot assays were performed to analyze intracellular pathways downstream of FGF signaling, namely Erk/Mapk and Akt/P13K. Grafted embryos were re-incubated for 4–8 hours and processed for immunoblot analysis. AER or ZPA explants were used as controls. Operated embryos were re-incubated for different time periods (15 minutes to 22 hours), collected in PBS and fixed for in situ hybridization.

**Results**

Limb hairy2 expression depends on Erk/MAPK and Aki/P13K pathway activation. The developing limb presents distinct domains of hairy2 expression (Fig. 1A), clearly visible from stages HH20 to HH28 (Pascoal et al., 2007). hairy2 expression oscillates with a 6 hour periodicity in the distal-most limb mesenchyme, including the ZPA (PPD, posterior positive domain) and is absent from both the proximal posterior region (PND, posterior negative domain) and the anterior limb (AND, anterior negative domain). In the distal-most limb mesenchyme (DCD, distal cyclic domain), hairy2 expression oscillates with a 6 hour periodicity (Pascoal et al., 2007) which, for the sake of simplicity, can be represented by three distinct phases (Fig. 1A–i).
hairy2 expression in these domains (Fig. 1Biii,iv; n=13/13). To interrogate if the observed effect was through FGF signaling, beads soaked in recombinant FGF8 or FGF2 were implanted in the DCD immediately following AER ablation, and hairy2 expression was assessed after 4 hours of incubation. Both FGF2 and FGF8 were able to maintain hairy2 expression around the beads (Fig. 1Bv,vi; FGF8: n=4/5; FGF2: n=3/3), supporting the requirement of FGFs for hairy2 expression in the distal limb mesenchyme. Accordingly, treating the distal limb field with FGF inhibitor SU5402 (n=12) down-regulated (n=7/12) or even abolished (n=4/12) hairy2 expression (Fig. 1Bvii,viii). FGF-mediated hairy2 regulation was further assessed by implanting FGF8-beads in different distal limb domains. hairy2 expression was enhanced in both DCD and PPD (Fig. 1Ci–iv; DCD: n=10/12; PPD: n=9/9) and up-regulated in the PND, a native hairy2 negative domain (Fig. 1Cv,vi; n=25/27). Contrastingly, FGF8 was unable to induce hairy2 in the AND (Fig. 1Cvii,viii; n=20/20), even after 20 hours of incubation and with increased amounts of FGF8 (supplementary material Fig. S1), indicating that the AND tissue is not competent to respond to FGF8 action on hairy2.

Erk/MAPK and Akt/Pi3K are two predominant intracellular pathways functioning downstream of AER/FGF signaling in the chick limb (Kawakami et al., 2003). The activation levels of each pathway upon implantation of FGF8-soaked beads either in the AND or PND were assessed by western-blot (Fig. 2A). FGF8-bead implantation in the PND increased p-Erk levels by 77% and p-Akt by 37% (Fig. 2Ai,iii), suggesting that FGF-induced hairy2 expression in the PND (Fig. 1Cv,vi) is mediated by Erk/MAPK and Akt/Pi3K pathways. This was also confirmed by co-implanting FGF8 with beads soaked in specific MAPK or PI3K inhibitors (U0126 and LY294002, respectively). In fact, the tissue facing the MAPK or PI3K inhibitor source no longer exhibited ectopic hairy2 expression (Fig. 2B) (U0126: n=4/4; LY294002: n=5/6). Contrastingly, FGF8-beads in the AND were unable to significantly activate Erk/MAPK or Akt/Pi3K pathways and even resulted in decreased p-Akt levels (Fig. 2Ai,iii). In these conditions, no ectopic hairy2 induction was observed (Fig. 1Cvii,viii), further substantiating Erk/Akt phosphorylation upstream of hairy2 induction.

Together, our results indicate that AER-derived FGF is absolutely required, although not sufficient, for hairy2 expression in the distal limb, mediated by Erk/MAPK and Akt/Pi3K signaling pathways. They also reveal that AER/FGF does not activate downstream signaling pathways in a uniform fashion along the limb AP axis. In fact, we observed higher Erk phosphorylation levels in the posterior limb, when compared to the anterior region of control limbs (Fig. 2Ai,ii).

**ZPA/SHH establishes the grounds for hairy2 expression through Gli3 activity modulation**

FGF8 was unable to induce hairy2 in the AND, although it readily induced ectopic hairy2 in the rest of the distal limb. This suggests the requirement of an additional signal, presumably present throughout all distal limb mesenchyme, except for the AND. SHH is a good candidate, since hairy2 wasn't impaired (Fig. 3Av,vi; fgf8: n=4/4; hairy2: n=22/27), strongly suggesting the requirement of ZPA-mediated signaling for hairy2 expression. Replacing the ZPA by QT6 cells constitutively secreting SHH rescued hairy2 (Fig. 3Av,vi; n=7/7), indicating that SHH is the ZPA-derived signal controlling hairy2 expression. Supporting these results, treatment with the SHH inhibitor cyclopamine abolished hairy2 in the distal limb.
Noticeably, SHH-beads positioned in the AND resulted in an anterior expansion of hairy2 expression (Fig. 3Biii,iv; n=11/15). These results clearly indicate that ZPA-derived SHH is essential for hairy2 expression in the distal limb mesenchyme.

Gli3 is a major signal transducer of SHH signaling, being present in an activator form (Gli3-A) when SHH is present which prevents its proteolytic cleavage to Gli3-Repressor (Gli3-R) (Wang et al., 2000). In agreement with this, SHH-bead implantation in the AND resulted in an accumulation of Gli3-A (up to 76% increase), at the expense of the Gli3-R form (Fig. 3C,D). In this condition, there was ectopic hairy2 induction (Fig. 3Biii,iv), contrarily to what was obtained when an FGF8-bead was implanted in the AND (Fig. 1Cvii,viii; supplementary material Fig. S1). FGF8 in the AND increased Gli3-R levels (Fig. 3C,D), suggesting that Gli3-R could be inhibiting hairy2. However, FGF8-bead in the PND also elevated the Gli3-R form (Fig. 3C,D) concomitantly with ectopic hairy2 induction (Fig. 1Cv,vi), indicating that hairy2 expression is not solely dependent on the presence or absence of Gli3-R. In fact, FGF8 in the PND increased both Gli3-R and Gli3-A to the same extent, evidencing that hairy2 expression relies on balanced Gli3-A/Gli3-R activities. Accordingly, Gli3-A/Gli3-R in the control limb is higher in the posterior than in the anterior region (Fig. 3E), which coincides with the presence and absence of hairy2 expression, respectively (Fig. 1A). This suggests the existence of a threshold of permissive Gli3-A/Gli3-R activity for hairy2 expression along the limb AP axis.

We then analyzed the correlation between the experimental conditions leading to hairy2 expression and Gli3-A/Gli3-R levels (Fig. 3E) and found a clear correlation between the presence/absence of hairy2 expression and a value of Gli3-A/Gli3-R higher/lower than one, respectively (Fig. 3E). An FGF8-bead in the AND increased the Gli3-R form and slightly diminished Gli3-A levels, overall decreasing the Gli3-A/Gli3-R ratio and failing to induce hairy2. SHH, on the other hand, greatly increased Gli3-A/Gli3-R in the AND with concomitant hairy2 induction. FGF8 in the PND maintained the Gli3-A/Gli3-R ratio of the posterior control limb and was able to induce ectopic hairy2. Together, these results show that SHH-mediated Gli3-A/Gli3-R>1 levels underlie the tissue’s ability to respond to FGF8 for hairy2 induction.
Instructive-FGF and permissive-SHH cooperatively underlie hairy2 expression in the distal limb mesenchyme

To further clarify the role of FGF and SHH on hairy2 expression, we started by interrogating the ability of FGF8 to induce hairy2 in the absence of the ZPA. FGF-beads implanted immediately upon ZPA ablation were still capable of locally inducing hairy2 expression around the bead (Fig. 4Ai,ii; n=5/5). However, if the ZPA-ablated limbs were incubated for 6 hours prior to FGF-bead implantation, FGFs were no longer able to induce hairy2 in neither the DCD (Fig. 4Aiii–iv; DCD: n=4/4) nor the PND (supplementary material Fig. S3). 6 hours was the incubation time previously found to be required for hairy2 down-regulation.
expression. Reinforcing this idea, SHH was incapable of inducing a distinct, instructive signal emanated by the AER on hairy2 defined as Gli3-A/Gli3-R

results suggest that SHH is creating a permissive condition plays an instructive role on hairy2 FGF influence (Fig. 3Biii,iv). It is thus highly unlikely that SHH expanding 6 hours of incubation. Here, SHH-beads were only capable of (SHH signaling.

Fig. 4. Limb hairy2 expression requires cooperative AER/FGF and ZPA/SHH signaling. (A) hairy2 expression obtained upon implantation of FGF8-beads in ZPA-ablated limbs. Implantation of FGF8-bead immediately after ZPA ablation induces local hairy2 expression (Aii, arrow), but fails to do so after 6 hours of ZPA ablation (Aiii,iv). (Bi,ii) hairy2 expression observed after co-implantation of FGF8 and SHH beads in the AND. Only the previously observed SHH-mediated expansion of hairy2 expression is obtained. FGF beads implanted following 6 hours of SHH treatment are capable of inducing ectopic hairy2 expression around the bead (Bi,iv, arrows). (Ci,ii) SHH-bead is unable to induce hairy2 upon AER ablation. SHH- and FGF8-beads are delimited by red and white dashed lines, respectively. Dorsal view; anterior to the top.

upon ZPA ablation (Fig. 3Aii,iv), possibly corresponding to the time it takes for Gli3-A/Gli3-R ratio to fall under 1 due to the lack of SHH signaling. Furthermore, ectopic SHH in the AND never up-regulated hairy2 locally around the bead even after 6 hours of incubation. Here, SHH-beads were only capable of expanding hairy2 anteriorly along the tissue under direct AER/FGF influence (Fig. 3Biii,iv). It is thus highly unlikely that SHH plays an instructive role on hairy2 expression. Instead, these results suggest that SHH is creating a permissive condition defined as Gli3-A/Gli3-R>1 (see previous section) for the action of a distinct, instructive signal emanated by the AER on hairy2 expression. Reinforcing this idea, SHH was incapable of inducing hairy2 in the AND upon AER ablation (Fig. 4Ciii,iv; n=3/3; supplementary material Fig. S3).

In a complementary approach, we set out to determine if a permissive state could be imposed on the AND, and allow FGF8-mediated hairy2 induction (supplementary material Fig. S1). When FGF8 and SHH-beads were concomitantly implanted in the AND, FGF8 did not induce hairy2 around the bead and the overall result (Fig. 4Bi,ii; n=5/5) was indistinguishable from that obtained with a SHH-bead alone (Fig. 3Biii,iv). Whereas, when FGF8-bead was implanted in a limb previously incubated for 6 hours with SHH, allowing for the establishment of tissue permissiveness (Fig. 3E), it now induced ectopic hairy2 (Fig. 4Biii,iv; n=6/6).

These experiments evidence that ZPA/SHH-mediated tissue permissiveness and AER/FGF instructive signals are both necessary for hairy2 expression. Moreover, by carefully analyzing the time frame required for FGF/SHH effect on hairy2 expression, we conclude that it does not involve a relay mechanism (supplementary material Fig. S4), but rather a convergence of signaling pathways in both time and space. The regulation of hairy2 expression is a fine example of temporal and spatial cooperative action of the developing limb signaling centers.

Distinct temporal and spatial properties of AER/FGF and ZPA/SHH in distal limb hairy2 expression regulation

To further understand the dynamics of hairy2’s dependence on AER/FGF and ZPA/SHH, a detailed study of the temporal response of hairy2 expression to the removal of each limb signaling center was performed. In the absence of the AER, hairy2 was down-regulated after 40 minutes (Fig. 5Ai,ii; n=2/2) and totally abolished upon 1 hour of incubation (Fig. 5Aiii,iv; n=5/5). This was not a consequence of cell death (supplementary material Fig. S5). Contrastingly, hairy2 expression was not affected even after 2 hours of ZPA ablation (Fig. 5Av,vi; n=9/10), and cyclopamine-mediated SHH signaling inhibition for 4 hours only mildly down-regulated hairy2 expression (supplementary material Fig. S2B). In fact, longer incubation periods were required for total hairy2 depletion.
in the distal mesenchyme after ZPA ablation (Fig. 5Avii,viii; n=22/27) or cyclopamine treatment (supplementary material Fig. S2B). This was not a consequence of impaired fgf8 expression (Fig. 3Ai,ii; supplementary material Fig. S2A). Together, these data evidence different temporal responses of hairy2 to both limb signaling centers: a short-term response to AER/FGF and long-term to ZPA/SHH.

To analyze the spatial effect of AER-mediated regulation on hairy2 expression we proceeded to ablate solely the posterior- or anterior-AER, randomly confirmed by fgf8 staining (Fig. 5Bi,ii,i,ix). Upon posterior-AER ablation, hairy2 was rapidly abolished in the PPD (Fig. 5Bv–viii; Phase1: n=4/4; Phase2: n=2/2), while shh expression was still present (Fig. 5Bi,iv; n=2/2). This was a spatially restricted effect, since hairy2 expression in the DCD remained unperturbed (Fig. 5Bvii,viii; n=2/2). Similarly, when the anterior-AER was ablated, hairy2 was unperturbed in the posterior limb and abolished in the DCD (Fig. 5Bxiii–xvi; Phase1: n=2/2; Phase2: n=5/5). As expected, shh expression was present in this condition (Fig. 5Bxii,xx; n=2/2). Together, these results evidence a restricted spatial response of hairy2 to AER signaling. On the contrary, ZPA ablation resulted in loss of hairy2 expression in the entire distal limb mesenchyme (Fig. 3Aiii,iv), indicating a long-range effect on hairy2 regulation. Replacement of the ZPA with QT6-SHH secreting cells, also rescued hairy2 expression pattern along the whole distal limb AP-axis, further supporting a long-range signaling mode of action for ZPA/SHH on hairy2.

Altogether, our data propose ZPA/SHH and AER/FGF signaling as distinct regulatory mechanisms acting on distal limb hairy2 expression, both temporally and spatially. SHH acts at a long-range and has a long-term permissive effect on hairy2, whereas the FGF effect is of a short-term, short-range instructive nature.

Discussion

The distal limb mesenchyme is under the influence of AER-derived FGFs and expresses appropriate FGF receptors (Sheeba et al., 2010). Applying complementary approaches, we found that AER/FGF8 plays an instructive role on limb hairy2 regulation. In agreement, FGFs have also been reported to induce HES genes in other systems, such as somitogenesis (Dubrulle et al., 2001; Niwa et al., 2007), inner ear development (Doetzlhofer et al., 2009) and other systems, such as somitogenesis (Dubrulle et al., 2001; Niwa et al., 2005) and Erk phosphorylation is reported to underlie HES-mediated Erk/Akt phosphorylation and, hence, hairy2 expression. Overall, we unveil Gli3-A/Gli3-R activity prevails (Gli3-A/Gli3-R<1). These observations suggest that SHH signaling is providing a permissive condition for FGFR-mediated Erk/Act phosphorylation and, hence, hairy2 expression. Nevertheless, it is tempting to speculate that hairy2 expression dynamics established by joint FGF/SHH signaling may play a role in limb digit patterning.

In the course of our analysis we also uncovered that FGF and SHH pathways present distinct temporal and spatial signaling properties on hairy2 expression in the distal limb, noticeably consistent with what would be expected for an instructive and permissive action, respectively. FGF8 impacted hairy2 in a short-term manner (as early as 45 minutes) while effect of SHH signaling alteration on hairy2 expression was only observed upon longer incubation periods (over 5 hours). In agreement, Harfe et al. showed that SHH-head implantation in the anterior limb alters Gli3-R levels only after 4 hours of incubation (Harfe et al., 2004). Besides distinct temporal signaling dynamics, AER/FGF and ZPA/SHH exhibit distinguishing spatial regulatory actions on limb hairy2 expression. We show that AER/FGF acts as a short-range signal on hairy2, possibly due to FGF interaction with heparin or heparan sulfate proteoglycans (Ornitz, 2000; Yu et al., 2009). Contrastingly, ZPA/SHH regulates hairy2 at a distance, which is consistent with SHH-mediated limb AP axis patterning through long-range diffusion from the ZPA (Harfe et al., 2004).
A positive feedback loop between FGF and SHH takes place during limb development (Zeller et al., 2009) and their joint requirement for the regulation of key molecules, such as 5’HoxD genes, is known (Laufier et al., 1994; Niswander et al., 1994; Yang et al., 1997). Our present work describes distal limb hairy2 as a unique molecular target of joint AER/FGF and ZPA/SHH action in both time and space. FGF/SHH-mediated regulation on hairy2 does not involve a relay mechanism, operating in a uniquely short-framed temporal window, while reflecting distinctive temporal and spatial properties of AER- and ZPA-derived signaling (Fig. 6). Overall, AER-derived FGF signaling through Erk/Akt, together with graded levels of fine-tuned SHH-mediated Gli3-A/Gli3-R activity, give rise to different hairy2 expression outcomes: persistent, cyclic or absent. Our results open the way for a better comprehension of a multitude of systems where FGF and SHH signaling pathways are involved and will further contribute to understand cyclic HES gene expression regulation in various processes that are also under joint FGF and SHH regulation, such as somitogenesis (Resende et al., 2010) neurogenesis (Shi et al., 2008; Sousa and Fishell, 2010) and stem cell pluripotency (Shi et al., 2008; Kobayashi et al., 2009).

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Competing Interests
The authors have no competing interests to declare.

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Fig. 6. Limb hairy2 expression as a temporal and spatial output of joint FGF and SHH signaling. (A) High levels of instructive AER-derived FGF signaling, together with the Gli3-A/Gli3-R>1 permissive state established by ZPA/SHH, define the required conditions for hairy2 expression. SHH acts as a long-term, long-range signal, ensuring a Gli3-A/Gli3-R>1 permissive state for hairy2 expression. In these conditions, short-term/short-range FGF signaling is capable of inducing hairy2 expression, acting as an instructive signal. (B) hairy2 is expressed in the mesenchyme adjacent to AER/FGF and presents distinct expression domains: hairy2 is persistently expressed in the posterior region overlapping the ZPA/SHH (PPD), is absent from the anterior limb (AND) and is cyclically expressed in the DCD. We describe FGF acting through Erk/MAPK and Akt/P3K as an instructive signal for hairy2 induction, in a short-term, short-range fashion throughout the distal limb mesenchyme. However, a permissive state mediated by SHH signaling is required for the tissue to respond to FGF inductive signal. SHH permissive signal is mediated by Gli3 activity and acts in a long-term, long-range manner and patterns hairy2 expression along the distal limb AP axis. PPD and DCD present Gli3-A/ Gli3-R above one (yellow dots), allowing sustained or oscillatory hairy2 expression. In the AND, AER/FGFs can no longer induce hairy2 since this tissue is in a non-permissive state: Gli3-A/Gli3-R below one (grey dots).
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