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Genetic evidence that FGFs play an instructive role in limb proximal-distal patterning

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Abstract

Half a century ago the apical ectodermal ridge (AER) at the distal tip of the tetrapod limb bud was shown to produce signals necessary for development along the proximal-distal (P-D) axis, but how these signals influence limb patterning is still much debated^{1,2}. FGF gene family members are key AER-derived signals^{3,4}, with *Fgf4*, *Fgf8*, *Fgf9*, and *Fgf17* expressed specifically in the mouse AER⁵. Here, we demonstrate that limbs lacking *Fgf4*, *Fgf9* and *Fgf17* have normal skeletal pattern, indicating that *Fgf8* is sufficient among AER-FGFs to sustain normal limb formation. Inactivation of *Fgf8* alone causes a mild skeletal phenotype^{6,7}, but when we also removed different combinations of the other AER-FGF genes we obtained unexpected skeletal phenotypes of increasing severity, reflecting the contribution each FGF can make to total AER-FGF signal. Analysis of the compound mutant limb buds revealed that in addition to sustaining cell survival, AERFGFs regulate P-D patterning gene expression during early limb bud development, thus providing genetic evidence that AER-FGFs function to specify a distal domain and challenging the long-standing hypothesis that AER-FGF signaling is permissive rather than instructive for limb patterning. We discuss how a two-signal model for P-D patterning can be integrated with the concept of early specification to explain the genetic data presented here.

Keywords

AER; apical ectodermal ridge; cell survival; FGF4; FGF8; FGF9; FGF17; limb development; proximal-distal patterning

Fgf8 is expressed in prospective AER cells of the nascent limb bud and subsequently throughout the AER until it regresses⁸, whereas *Fgf4*, *Fgf9*, and *Fgf17* expression commences after the AER is formed, is restricted to the posterior AER, and ceases at least a day before AER regression⁵ (Fig. 1a). When AER-FGFs are individually eliminated, only loss of *Fgf8* function perturbs skeletal patterning^{5-7,9-11}. The other AER-FGFs have been proposed to be essential, but functionally redundant components of a positive-feedback loop between the AER and the patterning center in posterior limb bud mesenchyme that produces Sonic hedgehog (SHH)^{5,12,13}. We tested this hypothesis by deleting *Fgf4* via Cre-mediated recombination in the AER of embryos homozygous for *Fgf9* and *Fgf17* null alleles^{10,11} (hereafter referred to as F4;9,17-triple knock-out [TKO] mutants; Fig. 1b). Because *Fgf4* deletion occurs before *Fgf4* expression normally commences⁵ (see Fig. 1a), the F4;9,17-TKO limb buds do not produce FGF4, or FGF9 or FGF17. Nevertheless, in F4;9,17-TKO skeletons (n=6), the three classically defined limb segments, stylopod (S, upper arm or leg), zeugopod (Z, lower arm or leg), and autopod (A, wrist/hand or ankle/foot), were essentially normally patterned (Fig. 1c).

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Consistent with this observation, *Shh* expression appeared normal in F4;9,17-TKO limb buds at embryonic day (E)10.5 by in situ hybridization (not shown) and qRT-PCR (Fig. 1d). Moreover, there was no compensatory upregulation of *Fgf8* in F4;9,17-TKO limb buds at E10.5 (Fig. 1d). These data demonstrate that *Fgf8* is sufficient for normal limb development, including sustaining *Shh* expression, and that whatever positive regulatory interactions occur between *Shh* and the posterior AER-FGF genes, they are dispensable for normal limb skeletal development.

Although not required when FGF8 is present, each posterior AER-FGF –FGF4, FGF9 and FGF17– may contribute to limb development. Such contributions can be uncovered by inactivating these genes, singly or in combination, along with *Fgf8* (refs. 14–15). To produce such double knock-out (DKO) and TKO mutants, we employed *Msx2-cre*, which functions earlier in hindlimb than in forelimb buds⁵ (see Fig. 1a). When inactivated by *Msx2-cre*, *Fgf8* is never expressed in hindlimb buds, but is transiently expressed in forelimb buds prior to E9.5; consequently, *Fgf8* knock-out (F8-KO) hindlimbs are more severely affected than forelimbs⁶. In F8;4-DKO mutants, the hindlimb skeleton fails to form whereas the forelimb skeleton develops but lacks many elements¹⁴. Likewise, in F8;4-DKO mutants lacking a copy of *Fgf9* (F8;4-DKO;*F9*^{-/+} mutants) there was no hindlimb, but a severely affected forelimb developed (see below). Compound mutant forelimbs thus provided a greater range of phenotypes for analysis than hindlimbs.

These phenotypes (summarized in Table S1) could be ranked in order of increasing severity. F8-KO (n=10), F8;17-DKO (n=8), and F8-KO;*F9*^{-/+} (n=6) mutants displayed a similar mild phenotype, with all skeletal elements present except for one digit, and slight hypoplasia of the stylopod and zeugopod (Fig 2a,b; data not shown; ref. 6). F8;9-DKO (n=16) and F8;9,17-TKO (n=8) mutants had the same defects, plus the zeugopod posterior element (ulna) was short and the anterior element (radius) was absent (Fig. 2c; data not shown). The hindlimb zeugopod was similarly affected in these mutants, but strikingly, it was the posterior zeugopod element (fibula) that was missing (not shown). Assays for *Sox9* expression, which marks the condensations that develop into skeletal elements¹⁶, showed that these patterning defects were detectable by E12.5 (Fig. 2h-j; data not shown), demonstrating that AER-FGFs are essential for establishing skeletal pattern at limb bud stages. A more severe phenotype was observed in F8;4-DKO mutants (n=5), in which the zeugopod consisted of only a hypoplastic ulna, and all autopod elements were absent except one or two phalanges (Fig 2d; ref. 14). Removing one copy of *Fgf9* further increased phenotype severity (n=14 mutants; Fig. 2e,f), and when both copies were removed, F8;4;9-TKO mutants (n=9) lacked all forelimb skeletal elements (Fig. 2g).

The simplest explanation for these data is that the individual AER-FGFs are functionally equivalent¹⁷, but differ in the extent to which they contribute to the AER-FGF signal, presumably reflecting differences in their temporal and spatial expression profiles, levels of expression, and binding specificities to FGF receptors in limb bud mesenchyme. If so, then the range of skeletal phenotypes observed as specific combinations of AER-FGFs are removed reflects a change in the level of total AER-FGF signal. In support of this hypothesis we found that at E10.5, the size/intensity of the expression domain of *Dusp6*, a downstream target of AER-FGF signaling¹⁸, negatively correlated with mutant phenotype severity (Fig. 2l-o). Together, the data suggest that *Fgf8* makes the greatest contribution to the AER-FGF signal, followed by *Fgf4*, *Fgf9*, and *Fgf17*. Furthermore, our data suggest that there is a critical threshold of AER-FGF signaling below which skeletal elements do not form.

The phenotype of the F8;4-DKO;*F9*^{-/+} forelimbs was especially striking: the stylopod (humerus) was present, but often shorter than normal and truncated distally, with elements of a single digit immediately distal to it. The most distal element usually had the pointed tip of a

terminal phalanx, occasionally with a nail overlying it (Fig. 2e,f; data not shown). Sometimes there was a substantial gap between humerus and phalangeal elements (Fig. 2f; Table S1). The complete absence of the zeugopod and most of the autopod was confirmed by *Sox9* expression assays at E12.5 (Fig. 2k; see Fig. S1 for data on *Hoxa11* expression). One possible explanation for this phenotype is that the severe reduction in AER-FGF signaling causes the death of most autopod and zeugopod progenitors in the early limb bud. However, consistent with previous reports^{14,15}, mesenchymal cell death in F8;4-DKO;F9^{-/+} mutants was detected in a proximal dorsal region at E10.5, and not distally, where autopod and distal zeugopod progenitors reside¹⁹. Cell death remained proximally restricted until E11.5, after which it was no longer detected (not shown). In mutant limb buds with more AER-FGF signaling, the cell death domain occupied a smaller percentage of limb bud volume (Fig 3a-d; data not shown). Because of where the dying cells were localized, it seems unlikely that autopod or zeugopod progenitor cell death is the main cause of the phenotype.

Another possible explanation for the lack of zeugopod and most of the autopod in F8;4-DKO;F9^{-/+} forelimbs is that AER-FGFs are involved in specifying distal cell fate, and when AER-FGF signaling is dramatically reduced, fewer cells are specified as distal. This hypothesis could be tested by examining the effects of reducing AER-FGF signaling on the expression of genes essential for P-D specification. *Meis1*, which encodes a homeobox transcription factor, is potentially one such gene because ectopic *Meis1* expression is reported to induce distal-to-proximal transformations in chicken limb buds²⁰. Initially, *Meis1* expression is detected throughout nascent limb bud mesenchyme; subsequently, a *Meis1*-negative distal domain is established and increases in size as the limb bud expands²⁰. Consistent with a role for FGFs in specification of a distal domain, FGF-bead implantation and drug inhibition studies showed that FGF signaling can repress *Meis1* expression²¹. Moreover, we previously found that in F8;F4-DKO limb buds the extent of the *Meis1*-negative expression domain was reduced¹⁴. However, because the F8;F4-DKO limb buds were considerably smaller than controls, it was impossible to know if the observed effect was secondary to the reduction in limb bud size.

Here, we were able to examine the effects of reducing AER-FGF signaling on *Meis1* expression independent of its effects on limb bud size, because the various AER-FGF compound mutant limb buds, although smaller than normal, were remarkably similar in overall size at E10.5 (Fig 3e-h; data not shown). One explanation for this curious finding is that their size at E10.5 reflects the elimination of cells that died due to inactivation of *Fgf8*, which is expressed from ~E9.0 and inactivated by ~E9.5, whereas the effects of loss of *Fgf4* and *Fgf9* function on cell survival are not yet evident because their expression begins later than *Fgf8*. By E11.5, though, size differences became evident among the compound mutants, due to extensive cell death and possibly a negative effect on cell proliferation after E10.5. These size differences were correlated with the decrease in AER-FGF signaling (not shown) and subsequently with skeletal phenotype severity (Fig. 2h-k and data not shown). Assays for *Meis1* at E10.5, when limb bud size was similar, showed that the *Meis1*-negative, distal domain was significantly reduced in F8;9-DKO and further reduced in F8;4-DKO;F9^{-/+} forelimb buds (Fig. 3i-l). To our knowledge, these data provide the first genetic evidence that AER-FGFs repress expression of a gene presumed to be involved in specification of proximal cell identity. In summary, we conclude that AER-FGF signaling serves at least two vital roles during limb development, to promote cell survival and to specify distal cell fate.

An important question is how our data fit with existing models for limb P-D patterning. The Progress Zone (PZ) model²² postulates that P-D pattern develops gradually, with cells in the distal limb bud mesoderm acquiring progressively more distal positional information over time, and that AER signals are not instructive for P-D patterning, but instead are “permissive,” keeping distal cells labile and able to change positional values by an unknown mechanism. However, our data showing that AER-FGF signaling is necessary to regulate *Meis1* expression

indicate that it functions as an instructive rather than permissive signal in limb development. Furthermore, the phenotypes of AER-FGF mutant skeletons (e.g. F8;4-DKO;F9^{-/+} forelimbs, which contain S and A but no Z elements) are not readily explicable by the Progress Zone model^{1,14}, which describes limb patterning as a progressive process whereby distal is specified only after proximal.

In contrast, the Early Specification model²³ postulates that cells along the limb P-D axis are specified to form the S, Z, and A limb segments at an early limb bud stage. Although not originally considered in this context, one model that can explain how such early specification occurs is the “two-signal model,” which proposes that limb bud cells are initially exposed to a proximal (P) signal from mesoderm flanking the limb bud, possibly retinoic acid (RA), and an opposing distal (D) signal (FGF) from the AER, which establish proximal and distal domains, respectively^{2,21}. Formation of a third (middle) domain might then occur as a result of interactions between cells at the boundary between proximal and distal domains over time (see Fig 4a), thus creating the three domains from which S, Z, and A segments will develop. Additional domains within the autopod, from which wrist elements, metacarpals, and digits develop, might likewise form as a result of cell-cell interactions at domain boundaries. This concept is consistent with the intercalation models proposed to explain amphibian limb regeneration^{24,25}. Early specification would thus be a “dynamic” process that takes place in concert with limb bud outgrowth. Our data support this model by providing genetic evidence that AER-FGFs function as an initial distal signal at early stages in limb development.

Such a two-signal dynamic specification model can easily explain the forelimb skeletal abnormalities reported here based on our findings that AER-FGFs have a dual function in limb development. Thus we suggest that in the AER-FGF mutants, the D-signal is reduced in proportion to the decrease in AER-FGF signal. Because there is less opposing D-signal, the P-signal, which is produced at the normal level, extends more distally than normal, specifying cells that would normally have formed distal elements as proximal and thereby compromising autopod and zeugopod development. However, the stylopod is not abnormally long because proximal cells die in the AER-FGF mutants (Fig. 4b-d; see Fig. S2a,b). This model of AERFGF dual function can also explain the limb skeletal defects observed in other studies of mouse mutants^{6,7,14} and X-irradiated chicken limb buds²⁶ in which the stylopod is severely reduced but distal elements are less affected (see Fig. S2c).

Presently, when segment specification occurs cannot be assessed and critical tests of this model cannot be performed because there are no molecular markers for the progenitors of the different segments. A promising avenue for identifying such markers has recently been opened by the demonstration that cells in proximal and distal regions of the early chicken limb bud are distinguishable by their sorting behaviors in vitro²⁷. It will be intriguing to determine whether FGFs produced in the AER are involved in establishing these differences.

METHODS SUMMARY

Production of mice

The mutant alleles employed in this study were maintained on a mixed genetic background. F4;9,17-TKO mutants were generated by crossing parental animals of the genotypes depicted in Figure 1b. Animals of the other genotypes were produced by generating and crossing appropriate parental mice. Control animals were offspring that did not inherit the *Msx2-cre* transgene.

Limb bud analysis

For quantitative Reverse Transcription PCR (qRT-PCR) cDNA was synthesized and analyzed using an ABI light cycler. For analysis of cell death, embryos were stained with LysoTracker

Red (Invitrogen) and sectioned. For each section, the LysoTracker Red-positive and limb bud areas were measured using tools in Adobe Photoshop. The *Meis1*-negative domain was determined for each sample by four independent, blind measurements that were then averaged.

Full methods and associated references are available in the online version of the paper at www.nature.com/nature.

METHODS

Production and analysis of mice

Strategies similar to that employed for generating the F4;9,17-TKO mutants (Fig. 1b) were used to generate animals of the other genotypes used in this study. For example, to generate F8;9-DKO or F8-KO;F9^{+/-} offspring, *Msx2-Cre*, *Fgf9*^{+/-}; *Fgf8*^{Δ2,3/fl} males were crossed with *Fgf9*^{+/-}; *Fgf8*^{fl/fl} or *Fgf8*^{fl/fl} females. F4;8;9-TKO or F4;8-DKO;F9^{+/-} animals were generated by crossing *Msx2-cre*, *Fgf9*^{+/-}; *Fgf4*^{Δ2,3/fl}; *Fgf8*^{fl/fl} males with *Fgf9*^{+/-}; *Fgf4*^{fl/fl}; *Fgf8*^{fl/fl} or *Fgf4*^{fl/fl}; *Fgf8*^{fl/fl} females. F8-KO and F8;F4-DKO animals were generated as previously described^{6,14}. Animals were genotyped by polymerase chain reaction (PCR) on DNA isolated from head or tail tissue, using primer pairs for *Msx2-cre*, *Fgf4* and *Fgf8* alleles¹⁴. For *Fgf9* we used the following primers: wt allele, F: 5'-GCAAGGGAGGGGAGTTGGATATACC-3' and R: 5'-GAAATCCAGTCCTGCAGTCAGCTGC-3'; mutant allele, F: 5'-CCTTTTCCCTCCTGTCTGCAAC-3' and R: 5'-TGTGCTCTAGTAGCTTTACGGAGCC-3'. For *Fgf17* the primers were: wt allele, F: 5'-GAAGTTTCTCCAGCGATGGG-3' and R: 5'-GACAGCAGAGAATCAATAGCTGC-3'; mutant allele, F: 5'-CCATGAGTGACGAACCTGG-3', R: 5'-TTGGCTTCTCTGGGACTCTACC-3'. Since homozygosity for *Fgf9* function causes perinatal lethality¹⁰, we assayed for skeletal pattern at E17.5 by staining cartilage and bone with alcian blue and alizarin red, respectively, using standard procedures. Embryos younger than E11 were staged by counting somites as previously described⁵. Whole-mount RNA in situ hybridization was performed according to a standard protocol.

Limb bud analysis

Limbs for quantitative Reverse Transcription PCR (qRT-PCR) were dissected and stored at -20° C in RNAlater (Qiagen, Cat# 1017980) until genotyping information was obtained. Individual limbs were then homogenized with the rotor-stator method and RNA was extracted (RNeasy micro kit, Qiagen, Cat# 74004, including the DNaseI step). cDNA was then synthesized (High Capacity cDNA kit, Applied Biosystems, Cat# 4368814) and analyzed with an ABI light cycler with the following primers: *cyclophilin*, F: 5'-TGGAGAGCACCAAGACAGACA-3' and R: 5'-TGCCGGAGTCGACAATGAT-3', *Fgf8*: F: 5'-TCTCCAGCACGATCTCTGTGAA-3' and R: 5'-GGAAGCTAATTGCCAAGAGCAA-3', *Shh*: F: 5'-AGCAGACCGGCTGATGACT-3' and R: 5'-AGAGATGGCCCAAGGCATTAA-3'. No-template controls and reverse transcriptase negative controls were included to detect contaminating genomic DNA. In addition, we performed control experiments in which we made cDNA from 1/2, 1, 2, and 4 limb buds to confirm that we could detect small differences in *Fgf8* and *Shh* expression.

Limb buds from embryos stained with LysoTracker Red as previously described³¹, were embedded in 4% low-melt agarose and sectioned (25μm) on a Leica vibrating microtome. Serial transverse sections were collected, counterstained with DAPI, mounted on slides, and photographed on a Zeiss Axiophot fluorescence microscope or a Nikon Spectral confocal microscope at the UCSF Nikon Imaging Center. For each section, the LysoTracker-positive area was measured by setting a black/white threshold level in the image of the limb bud in that section and counting white pixels in the mesenchyme. Area was measured by counting the total

number of pixels in the image of the limb bud. Data from each section was summed across the sample (voxels) to obtain the total amount of cell death and the size (volume) of each limb bud.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *Fgf8* is sufficient for normal limb development

(a) Schematic diagram illustrating temporal aspects of AER-FGF gene expression and the stages at which the *Msx2-cre* transgene functions to inactivate *Fgf8* and *Fgf4* floxed alleles in the AER. Note that development of forelimb buds, as marked by *Fgf8* expression, commences before that of hindlimb buds, that *Fgf8* expression precedes that of *Fgf4*, *Fgf9*, and *Fgf17*, and that *Msx2-cre* functions earlier in hindlimb than in forelimb buds^{5,6}. (b) Schematic diagram of mouse chromosome 14 showing the map positions of *Fgf9*, *Fgf17*, and the *Msx2-cre* transgene, which lies within 1 centimorgan (cM) of *Bmpr1a* (not illustrated), 12.8 cM from the centromere (circle)²⁸. Because of this linkage, once the parental animals (male on left; female on right) were generated we could produce progeny of the genotype illustrated, in which the *Fgf4* conditional null allele (*Fgf4^{fl}*) is inactivated by *Msx2-cre* function in the AER (*Fgf4;Fgf9,Fgf17* TKO mutants) at a frequency of 12.5% ($n=6/48$), close to the expected frequency of 15.5%. (c) *Fgf4;Fgf9,Fgf17* TKO forelimb (FL) and hindlimb (HL) skeletons at E17.5, which are indistinguishable from wild-type (not shown) except for an enlarged deltoid tuberosity caused by loss of *Fgf9* function after condensation²⁹. (d) qRT-PCR assays for *Fgf8* and *Shh* expression. A representative experiment on forelimb buds from embryos at ~E11.0 (39–40 somites; $n=4$ limb buds for each genotype) is shown. Values are normalized to cyclophilin expression and are shown as means $\pm$ the standard deviation. The difference between control and mutant limb buds with respect to *Fgf8* and *Shh* expression was not significant (*Fgf8*, $p=0.61$; *Shh*, $p=0.80$). A two-tailed Student's t-Test was employed, using the average of triplicate cycle count values for each limb bud. Similar results were obtained for limb buds at 33, 34, 35, and 37 somites. **Abbreviations:** A, autopod; DT, deltoid tuberosity;

Fe, femur; Fib, fibula; Hu, humerus; Ra, radius; Sc, scapula; S, stylopod; som, somite number; Ti, tibia; Ul, ulna; Z, zeugopod; I-V, digit numbers from anterior to posterior.

Figure 2. Effects of inactivating AER-FGF genes on skeletal development

(a-g) Comparison of skeletal preparations of forelimbs from E17.5 embryos of the genotypes indicated. Asterisk (panels b, c and i) indicates that the mutant autopod has only 4 digits. Open triangle (panel c) indicates the lack of the anterior element (radius). The differences in humerus thickness/shape and deltoid tuberosity size among mutants of the various genotypes illustrated in panels b-d were also observed among mutants of the individual genotypes, suggesting they are due to background genes. (e and f) Two examples of *Fgf8;Fgf4*-DKO;*Fgf9*^{-/-} forelimb skeletons, illustrating the more and less common phenotypes, respectively. The insets show the distal element of the limbs at higher magnification. The bracket (panel f) indicates the gap between the digit-like element and the distal end of the humerus. (h-o) Expression of *Sox9* at E12.5 and of *Dusp6* at E10.5 (37 somites), as detected by RNA in situ hybridization in whole mount in forelimb buds from embryos of the genotypes indicated. *Sox9* expression marks the

condensations that prefigure the skeletal elements. The dotted white lines (panels j and k) outline the condensations that will develop into the scapula. Note the absence of the developing radius (panel j). The bracket (panel k) demarcates the region devoid of *Sox9*-positive cells between the developing condylar portion of the humerus and a distal condensation that presumably represents the distal part of a digit. **Abbreviations**, as in legend to Fig. 1, and pRa, prospective radius; pUl, prospective ulna.

Figure 3. Effects of inactivating AER-FGF genes on cell survival, limb bud size, and *Meis1* expression

(a-c) Confocal images of sections through E10.5 forelimb limb buds (35–36 somites) of the genotypes indicated stained with LysoTracker Red, which labels apoptotic cells as well as healthy cells engulfing apoptotic debris³⁰, and DAPI (blue). (d) Graph showing the percent of the limb bud volume that is LysoTracker-positive for each genotype. The difference between the two mutants was statistically significant ($p=0.014$, two-tailed Student's t-Test). (e-g) Dorsal views of E10.5 forelimb buds (36–37 somites) from embryos of the genotypes indicated. (h) Graph showing the total volume of E10.5 limb buds (35–37 somites) of the genotypes indicated. No statistically significant difference between mutants was detected ($p=0.79$, two-tailed Student's t-Test). (i-k) E10.5 forelimb buds (37 somites) were assayed by RNA in situ hybridization in whole mount for *Meis1* expression. The dashed tan and solid yellow brackets in each panel demarcate the *Meis1*-positive proximal and *Meis1*-negative distal domains in the limb bud mesenchyme, respectively. The length of each bracket reflects the average of the four

measurements made on each sample. (I) The percent of total limb bud proximal-distal (P-D) length (i.e. sum of the lengths of the proximal and distal domains) that is *Meis1*-negative is shown for each limb bud. The difference between mutants was statistically significant ($p=0.0003$, two-tailed Student's t-Test using the average measurements obtained for each limb bud). **Abbreviations:** A<->P, anterior-posterior; AER, apical ectodermal ridge; D<->V, dorsolventral; P<->D, proximal-distal. Error bars in graphs show the standard deviation.

Figure 4. AER-FGF mutant phenotypes can be explained by a two-signal dynamic specification model for limb proximal-distal patterning

(a) Diagrams illustrating: (left) the concept that a proximal (P) signal(s) from the mesoderm flanking the limb bud and opposing distal (D) signals from the AER (FGFs) specify limb bud P and D domains^{2,21} depicted as tan and purple boxes, respectively, by E10.5; (center) the hypothesis, based on recent fate mapping studies in chicken limb buds suggesting a common progenitor for zeugopod and autopod cells¹⁹, that a middle domain (purple and gold cross-hatching) forms within the D domain adjacent to the P domain, possibly as a consequence of cell-cell interactions; (right). Three domains develop into the three classically defined segments: stylopod (S, tan), zeugopod (Z, gold), autopod (A, purple).

(b) Diagrams representing wild-type and AER-FGF mutant forelimb buds in dorsal view at E10.5 and E11.5. Note that at E10.5, all mutant limb buds are equal in size but smaller than wild-type (quantified in this study for all genotypes except F8;4-DKO); by E11.5 mutant limb bud size is reduced in proportion to the reduction in AER-FGF signal. At E10.5, dying cells (Fig. 3a-d; data not shown; ref. ¹⁴) are represented by red dots; cell death in the AER is not depicted. By E11.5, dying cells are no longer observed in the mutant limb buds.

(c) *Meis1*-positive P and *Meis1*-negative D domains, represented by tan and purple boxes, respectively, can be compared between mutants because the limb buds are the same size (see Fig. 3 e-h and text). Tan and purple circles represent the opposing P- and D-signals, respectively. In the mutants, the P-signal remains the same as in wild-type limb buds, while the D-signal decreases in proportion to the AER-FGF signal. Arrows indicate the extent of

limb bud P-D length that is under the influence of these signals. Note that in mutants the P-signal extends over a region that, in wild-type limb buds, would normally be exposed to D-signal. This occurs both because the limb bud is reduced in size due to loss of cells specifically in the P domain and because the D-signal is much weaker than normal. Consequently, cells that would have been fated to form autopod or zeugopod if the AER-FGF signal were normal, are specified as proximal and develop into stylopod.

(d) Schematic diagrams representing the size and number of elements present in forelimbs of each genotype at birth. In F8;9-DKO limb buds, enough cells were specified as distal to form most Z and A elements, and S was only slightly reduced in length due to proximal cell death before and after E10.5. In F8;4-DKO;F9^{-/+} limb buds, the distal domain was reduced in size such that only the distal-most A and no Z elements formed, and the S was substantially reduced in length due to proximal cell death before and after E10.5.