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Pitx1 is necessary for normal initiation of hindlimb outgrowth through regulation of Tbx4 expression and shapes hindlimb morphologies via targeted growth control

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SUMMARY

The forelimbs and hindlimbs of vertebrates are morphologically distinct. Pitx1, expressed in the hindlimb bud mesenchyme, is required for the formation of hindlimb characteristics and produces hindlimb-like morphologies when misexpressed in forelimbs. Pitx1 is also necessary for normal expression of Tbx4, a transcription factor required for normal hindlimb development. Despite the importance of this protein in these processes, little is known about its mechanism of action. Using a transgenic gene replacement strategy in a *Pitx1* mutant mouse, we have uncoupled two discrete functions of Pitx1. We show that, firstly, this protein influences hindlimb outgrowth by regulating *Tbx4* expression levels and that, subsequently, it shapes hindlimb bone and soft tissue morphology independently of *Tbx4*. We provide the first description of how Pitx1 sculpts the forming hindlimb skeleton by localised modulation of the growth rate of discrete elements.

KEY WORDS: Pitx1, Limb-type morphology, Tbx4, Mouse

INTRODUCTION

The forelimb and hindlimb are serially homologous structures. The patterning of the forming limb is controlled by signalling molecules expressed in key signalling centres and their targets (Duboc and Logan, 2009; Zeller et al., 2009; Butterfield et al., 2010), which are thought to act equivalently in both forelimb and hindlimb buds. Cells in the forelimb and hindlimb interpret this common signalling input to form morphologically distinct limb elements. Very little is known about how these distinct limb type morphologies are achieved. Classical experiments performed in the chick demonstrate that forelimb- or hindlimb-forming potential is specified prior to limb bud development in an autonomous manner. Cells transplanted from a wing-forming region give rise to a wing when grafted to an ectopic location and, conversely, comparable leg grafts develop into a leg (Hamburger, 1938; Stephens et al., 1989; Saito et al., 2002; Saito et al., 2006).

The paired-type homeodomain transcription factor Pitx1 is the only protein that has been unambiguously implicated in determining limb-type morphologies. Pitx1 is expressed in the hindlimb-forming region and hindlimb bud mesenchyme, but not in the forelimb. Functional studies performed in both chick and mouse support a role for Pitx1 in determining hindlimb morphology. When misexpressed in the developing chick wing, elements of the wing adopt some characteristic leg features (Logan and Tabin, 1999; Takeuchi et al., 1999). *Pitx1* misexpression in the mouse forelimb results in transformation and translocation of specific muscles, tendons and bones to acquire a hindlimb-like morphology (DeLaurier et al., 2006). Consistent with these observations, in *Pitx1*-null mice, the hindlimb skeleton loses some of its characteristic features (Lanctot et al., 1999; Szeto et al., 1999; Marcil et al., 2003). For example, the diameter of the wild-type fibula is around half that of the tibia whereas the homologous elements in the forelimb zeugopod (ulna and radius) are roughly equivalent in diameter. In the *Pitx1* mutant, the fibula and tibia have equivalent diameters. The knee joint also lacks a patella and the size and shape of the calcaneus bone in the ankle are abnormal. The mechanisms by which *Pitx1* normally modulates the establishment of these hindlimb morphologies remain unknown.

Tbx4 and *Tbx5* encode paralogous T-box transcription factors that are expressed exclusively in the hindlimb and forelimb, respectively. Initially based on this restricted expression pattern, they were proposed as candidates to determine limb-type morphologies (Gibson-Brown et al., 1998; Isaac et al., 1998; Ohuchi et al., 1998). Loss-of-function experiments in mouse and zebrafish have demonstrated that Tbx5 and Tbx4 play crucial, conserved roles in forelimb and hindlimb bud initiation, respectively (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002; Hasson et al., 2007). In mouse embryos lacking or with conditional deletion of *Tbx5*, the forelimb buds fail to form (Agarwal et al., 2003; Rallis et al., 2003). In the absence of *Tbx5*, *Fgf10* expression is not initiated and, as a result, the fibroblast growth factor (FGF) signalling loop between limb mesenchyme and ectoderm, which is essential for limb bud formation and continued limb outgrowth, is never established (Duboc and Logan, 2011). *Fgf10* appears to be a direct target of *Tbx5* (Ng et al., 2002; Agarwal et al., 2003). An equivalent regulatory relationship exists between *Fgf10* and *Tbx4* in the hindlimb, although *Tbx4* is not required exclusively for *Fgf10* to be expressed. Consequently, in *Tbx4*-null mice, a hindlimb bud does form, but it is drastically reduced in size owing to disrupted initiation and maintenance of *Fgf10* expression (Naiche and Papaioannou, 2003).

There has been controversy in the literature regarding the function of Tbx5 and Tbx4 in determining limb-type morphologies. Ectopic expression of *Tbx5* in the developing chick hindlimb bud has been reported to partially transform the morphology of the leg to a more wing-like type (Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999). Conversely, *Tbx4* misexpression in the forelimb can

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apparently partially transform the wing causing it to develop some hindlimb characteristics (Takeuchi et al., 1999). These observations are in contrast with results from the mouse. Following conditional deletion of *Tbx5* and simultaneous replacement with transgenically supplied *Tbx4*, forelimb outgrowth is rescued (Minguillon et al., 2005; Minguillon et al., 2009). These results demonstrate that the hindlimb-expressed gene *Tbx4* is sufficient to compensate for *Tbx5* in forelimb initiation and that forelimb morphologies can form in the absence of *Tbx5* and presence of *Tbx4*. Taken together, these results demonstrate that, in mouse, Tbx5 and Tbx4 share common roles during limb initiation but do not determine limb-type morphologies.

Pitx1 is required for normal levels of *Tbx4* expression in the hindlimb (Lanctot et al., 1999; Szeto et al., 1999) and misexpression of *Pitx1* in the forelimb of the chick or the mouse is sufficient to induce ectopic *Tbx4* expression (Logan and Tabin, 1999; DeLaurier et al., 2006). A crucial point that has been overlooked in previous studies is that some aspects of the *Pitx1*$^{-/-}$ hindlimb phenotype can be attributed to hypomorphic levels of *Tbx4* transcripts that will lead to the abnormal development of hindlimb structures. In this study, we have uncoupled two functions of Pitx1. First, Pitx1 influences hindlimb outgrowth by regulating *Tbx4* expression and, second, Pitx1 shapes hindlimb bones and soft tissues independently of Tbx4 activity. We identify Pitx1 as a regulator of hindlimb bone and soft tissue morphology. Furthermore, we provide the first explanation of Pitx1 mode of action during the emergence of hindlimb morphologies by showing how this gene increases the growth rate of discrete bones, the metatarsals, in the forming appendicular skeleton.

MATERIALS AND METHODS

Embryos and mouse lines

Mouse embryos were staged according to Kaufman (Kaufman, 2001). Noon on the day a vaginal plug was observed was taken to be embryonic day (E) 0.5. *Pitx1*$^{-/-}$ line was kindly provided by Dr M. G. Rosenfeld (Szeto et al., 1999). *Prx1-Pitx1* (Minguillon et al., 2005), *Prx1-Tbx4*, *Prx1-Tbx5* and chimeric transgenic lines have been previously described (Minguillon et al., 2009). The *Scx*-GFP reporter line was kindly provided by Dr Ronen Schweitzer (DeLaurier et al., 2006; Pryce et al., 2007).

In situ hybridisation

Whole-mount and section in situ hybridisation protocol and *Tbx4* and *Pitx1* probes have been described previously (Riddle et al., 1993; DeLaurier et al., 2006).

Whole-mount immunohistochemistry

Immunohistochemistry was performed as previously described (DeLaurier et al., 2006) and analysed by confocal microscopy (LSM5 Pascal, Zeiss). Skeletal elements, muscles and tendons were identified as previously described (DeLaurier et al., 2006) and using the mouse limb anatomy atlas (Delaurier et al., 2008) (http://www.nimr.mrc.ac.uk/3dlimb/).

Skeletal preparations

The cartilage and bone elements of mouse embryos were stained with Alcian Blue and Alizarin Red, respectively, essentially as described (Hogan et al., 1994).

Cell culture and luciferase assay

Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). The *Tbx4* and *Tbx5* cDNAs (Minguillon et al., 2009) cloned into pCDNA3.1 expression plasmid (Invitrogen) were transfected using Lipofectamine (Sigma), with either pGL3-braBS or PGL3-Fgf10-P firefly luciferase reporter (Rallis et al., 2003) into NIH-3T3 mouse fibroblasts, at 70% confluence 24 hours after seeding (660 ng of total DNA per well). Measurements were performed following manufacturer's instructions using an Anthos Lucy2 Microplate Luminometer.

Skeletal measurement and analysis

Measurements were taken using a stereo microscope equipped with a graticule eyepiece. Skeletal elements of each limb were measured as follows. Pelvis length was measured from the head of the ilium to the bottom of the ischium. Femur length was taken as the ossified portion of the bone shaft and femur diameter at the mid-shaft of the bone. Similarly, tibia length was taken as the ossified portion of the bone shaft, and tibia and fibula diameter at the mid-shaft of the bone. The length of the autopod was measured from the tip of the calcaneus to the tip of the third phalanx of digit three. A total of 140 limbs were measured. For each transgenic line used to rescue the *Pitx1*$^{-/-}$ background, a number of limbs ($n \geq 12$) were measured, derived from at least three separate litters. The average measurement for each condition (wild type, *Pitx1*$^{-/-}$, and transgenic rescue of the *Pitx1*$^{-/-}$ background) was normalised to the average of the wild-type value ($n \geq 4$) for each separate litter to allow comparison and account for the biological variability across distinct litters. The histogram (Fig. 2B) shows the mean of normalised values ± s.e.m. $*P<1\times10^{-4}$; $**P<5\times10^{-5}$, calculated by Student's *t*-test. Metatarsal and metacarpal measurements: For each condition, $n \geq 8$, derived from at least two different litters. Metacarpals/metatarsals were measured from the proximal junction with the carpal/tarsal bone to the distal joint with the first phalange of the third digit. Values represent the mean of the measured size for each condition ± s.d. (Fig. 2B).

RESULTS

The outgrowth defect in *Pitx1*$^{-/-}$ hindlimbs can be rescued by *Tbx4* or *Tbx5*

Pitx1$^{-/-}$ mutant hindlimbs display a complex series of skeletal defects affecting both size and morphology of the bones. *Tbx4* is required for initiation of hindlimb bud outgrowth, although subsequent outgrowth of the hindlimb is independent of this factor (Naiche and Papaioannou, 2007). *Pitx1* is necessary for normal levels of *Tbx4* transcription (supplementary material Fig. S1) (Lanctot et al., 1999; Szeto et al., 1999). Therefore, to understand which aspects of the *Pitx1*$^{-/-}$ phenotype can be attributed to hypomorphic levels of *Tbx4* expression, we took a gene replacement approach exploiting the *Prx1* (*Prrx1* – Mouse Genome Informatics) gene regulatory element that is capable of driving gene expression in the developing limb buds (Martin and Olson, 2000). *Prx1*-driven transgenes, expressing either *Pitx1*, *Tbx4*, *Tbx5* or chimeric forms of *Tbx4*/*Tbx5*, were used to replace endogenous *Pitx1* expression in the hindlimb. We then carried out a quantitative analysis of the skeletal defects in the mutant hindlimbs by a systematic measurement of affected bone elements and compared these with the wild-type hindlimb to uncover the ability of the different transgenes to rescue hindlimb outgrowth and morphology. Consistent with previous descriptions (Lanctot et al., 1999; Szeto et al., 1999), *Pitx1*$^{-/-}$ hindlimbs are shorter overall compared with control littermates (Fig. 1A,G). This is a consequence of a general reduction in length and width of all the hindlimb long bones (femur, tibia and fibula) (Fig. 1A,G, Fig. 2B). As anticipated, *Prx1-Pitx1* rescued hindlimbs are almost indistinguishable from wild type in overall size and morphology (compare Fig. 1A,J and Fig. 2B). This indicates that the *Prx1* promoter can drive sufficient transgene expression levels and in an appropriate time frame to rescue the mutant phenotype. We have shown previously that *Prx1-Tbx4* and *Prx1-Tbx5* as well as *Tbx4/5* chimeric transgenes (*M5N* and *M4*C; Fig. 2A) are sufficient to compensate for the conditional deletion of *Tbx5* from the presumptive forelimb (Minguillon et al., 2005; Minguillon et al., 2009). Moreover, *Prx1-Tbx4* is sufficient to rescue normal hindlimb formation after conditional deletion of *Tbx4* (data not shown). This shows that these different transgenes are providing physiologically relevant levels of the proteins. *Pitx1*$^{-/-}$ embryos have a shortened pelvis that is, on average, only

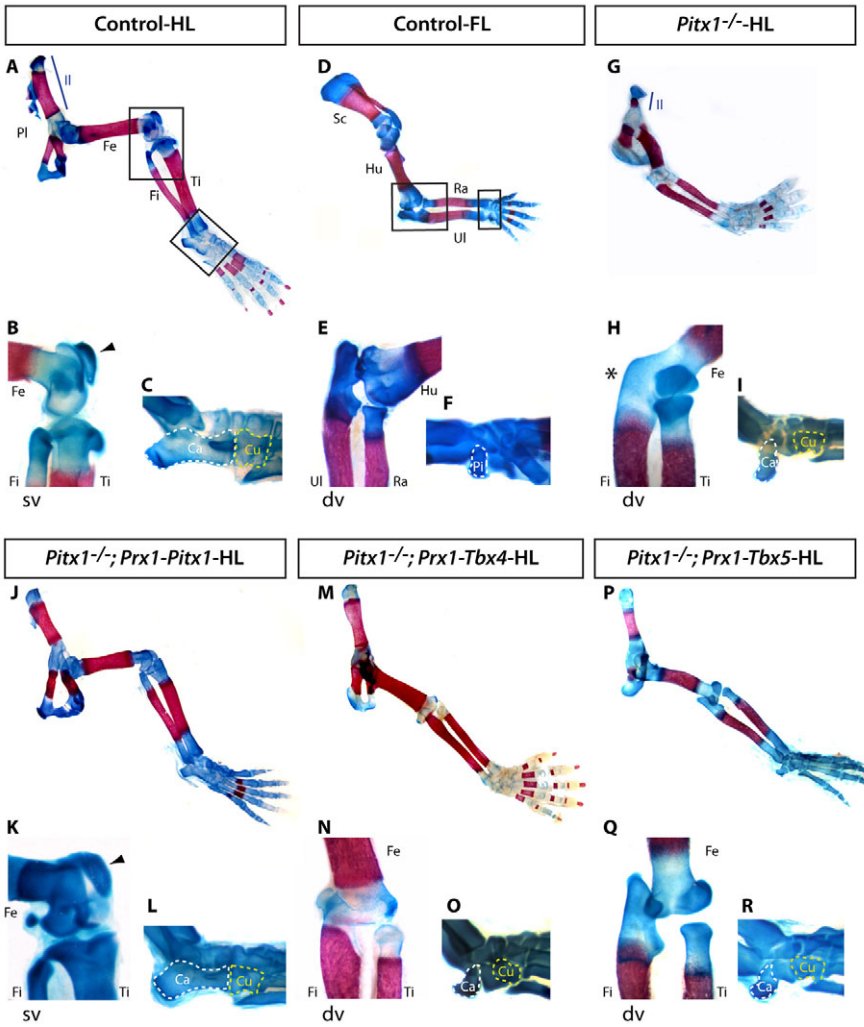

Fig. 1. Comparison of skeletal preparations of *Pitx1*$^{-/-}$ mutant hindlimbs and hindlimbs rescued with either *Pitx1*, *Tbx4* or *Tbx5* transgenes. (**A-R**) Preparations of E17.5 mouse limb skeletons for (A-C) control hindlimb (HL), (D-F) control forelimb (FL), (G-I) *Pitx1*$^{-/-}$ hindlimb, (J-L) *Pitx1*$^{-/-}$;*Prx1-Pitx1* hindlimb, (M-O) *Pitx1*$^{-/-}$;*Prx1-Tbx4* hindlimb and (P-R) *Pitx1*$^{-/-}$;*Prx1-Tbx5* hindlimb. For each condition, three panels containing the entire limb skeleton, magnification of knee/elbow and ankle/wrist (both boxed in A,D) are shown. In magnifications of the ankle region, the calcaneus (ca, white dashed line) and cuboid (cu, yellow dashed line) or homologous region of the FL comprising the pisiform (Pi, white dashed line) are outlined. Positions of the pelvis (Pl), ilium (Il), femur (Fe), humerus (Hu), fibula (Fi), tibia (Ti), radius (Ra) and ulna (Ul) are labelled. The arrowhead indicates the presence of a patella in the control knee (B) that is rescued in the mutant by the *Prx1-Pitx1* transgene (K). Fusion of the femur and fibula in the *Pitx1*$^{-/-}$ mutant is marked with an asterisk (H). Orientation of the knee/elbow panels are indicated as follows: sv; side view, dv; dorsal view.

63% of the wild-type length at E17.5 and have a truncated or absent ilium (Il, Fig. 1G). Pelvis length is rescued to 95% of the wild-type size by a *Prx1-Tbx4* transgene (Fig. 1M, Fig. 2B). These data show that the failure of proper growth of the pelvis in *Pitx1*$^{-/-}$ embryos is due to hypomorphic levels of *Tbx4* and demonstrates that the formation of the pelvic girdle is a *Tbx4*-dependent process and that it can form in the absence of *Pitx1* activity. Growth defects in long bones, such as shortening of the femur (68% of normal length) as well as decrease in femur width (81% of normal width) and shortening of the tibia (75% of normal length), are also rescued by the *Tbx4* transgene (Fig. 2B). These data demonstrate that the size defects of long bones in the *Pitx1*$^{-/-}$ mutant hindlimb are caused by lowered (hypomorphic) levels of *Tbx4* expression. Significantly, Tbx5 or the chimeric Tbx4/5 proteins M5N and M4C are equally able to compensate for the reduction in *Tbx4* levels (Fig. 1P-R, Fig. 2B; supplementary material Fig. S2) consistent with a model in which *Tbx4* and *Tbx5* perform equivalent roles in hindlimb and forelimb outgrowth, respectively.

Although both *Tbx4* and *Tbx5* are able to rescue limb outgrowth defects in the absence of *Pitx1*, the *Prx1-Tbx4*, *Prx1-M4C* and *Prx1-M5N* transgenes rescue slightly more effectively than the *Prx1-Tbx5* transgene (Fig. 2B). The differences in potencies observed between the different transgenes could simply reflect transgene expression levels, but could also indicate a difference in the transcriptional activities of Tbx4 and Tbx5. To help distinguish between these two possibilities, we carried out luciferase assay analysis using Tbx-responsive elements linked to a luciferase reporter, in the presence of either Tbx4 or Tbx5. A 2.4-fold activation of luciferase activity was observed compared with control. The results, however, show no statistical difference between the induction potency of Tbx4 and Tbx5 using Student's *t*-test, suggesting that both are equivalently potent transcriptional activators in this assay (Fig. 2C). This is consistent with their proposed roles in the positive regulation of *Fgf10* transcription. Therefore, the differences in the extent of rescue observed between the different transgenes most likely reflect the levels and/or timing of transgene expression rather than any significant biological differences in the activities of Tbx4 and Tbx5 proteins.

Hindlimb morphological characteristics are not rescued by *Tbx4* or *Tbx5*

In the *Pitx1*$^{-/-}$ hindlimb, many of the morphological characteristics of the hindlimb skeleton fail to form. For example, in the knee, the patella bone is absent (compare Fig. 1B and 1H) and the tibia and fibula bones lose the hindlimb characteristic difference in diameter (compare Fig. 1A and 1G). Normally, the fibula is positioned behind the tibia (Fig. 1B). In the *Pitx1*$^{-/-}$ mutant, bones at the knee are misplaced and the fibula is found lateral to and fused with the head of the femur (Fig. 1G,H). *Tbx4* and *Tbx5* transgenes, in the

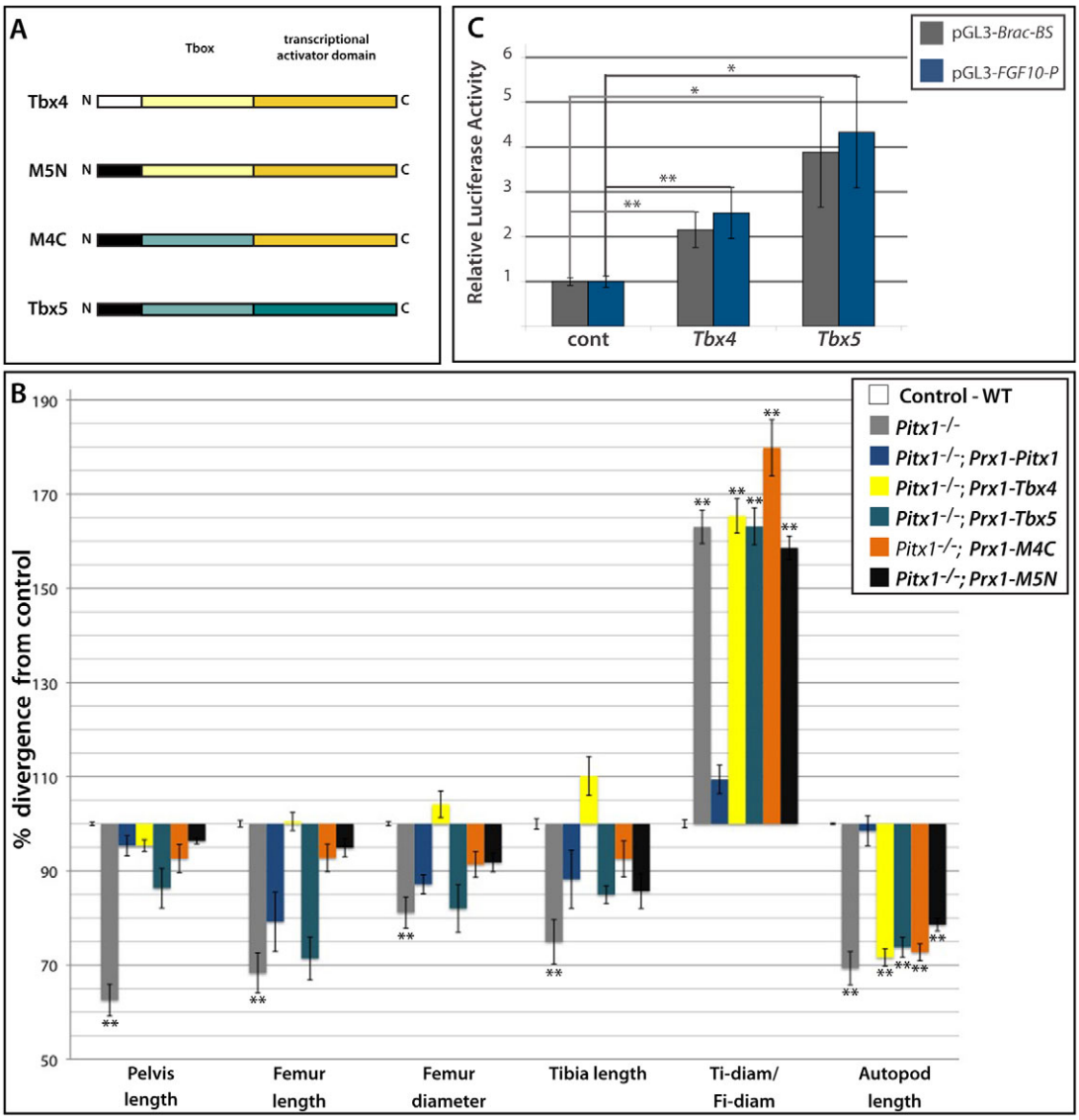

Fig. 2. Outgrowth defects can be rescued by *Tbx4* or *Tbx5* in the absence of *Pitx1* expression but *Tbx4* and *Tbx5* do not contribute to shaping hindlimb morphology. (**A**) Schematic of the different chimeric Tbx4/Tbx5 proteins. White, Tbx4 N-terminal domain; yellow, Tbx4 DNA-binding (T-box) domain; orange, Tbx4 transcriptional activator domain; black, Tbx5 N-terminal domain; pale green, Tbx5 DNA-binding (T-box) domain; dark green, Tbx5 transcriptional activator domain. (**B**) Quantification of the efficiency of rescue of skeletal defects by the different transgenes. Histogram represents the percentage divergence from wild-type control ± s.e.m. $*P<1\times10^{-4}$, $**P<5\times10^{-5}$, determined by Student's *t*-test. Hindlimb outgrowth measured by pelvis, femur, tibia length and femur diameter can be rescued by *Pitx1* and *Tbx4/5* transgenes, whereas hindlimb morphology, assayed by tibia/fibula diameter ratio and autopod length, is rescued by the *Pitx1* transgene but none of the *Tbx* transgenes. (**C**) Relative luciferase activity measured in NIH-3T3 cells transfected with mouse *Tbx4* or *Tbx5* expression vectors, together with a brachyury-binding site firefly luciferase (pGL3-Brac-BS) reporter or a fragment of the mouse *Fgf10* promoter encompassing the *Tbx5* response element (pGL3-FGF10-P) and a normalisation plasmid. Luciferase activity is shown relative to the control ± s.e.m. $*P<0.05$, $**P<10^{-3}$, determined by Student's *t*-test.

background of the *Pitx1*$^{-/-}$ mutant, are able to rescue the size of the long bones; however, they cannot rescue patella formation, which is absent at the knee joint (Fig. 1N,Q). The ratio of tibia/fibula diameter remains the same as that observed in the mutant (compare Fig. 1G with 1M,P; Fig. 2B) and the fibula remains inappropriately positioned lateral to the femur head (compare Fig. 1H with 1N,Q). There is also a striking alteration in the tarsal morphology in the *Pitx1*$^{-/-}$ ankle; for example, the calcaneus bone (Ca; Fig. 1C) is smaller and has an abnormal shape (Fig. 1I). Another hindlimb characteristic is the relative elongation of the autopod compared with the forelimb autopod (Fig. 1A,D). In the *Pitx1*$^{-/-}$, mutant the autopod is shorter (Fig. 1G). Neither formation of a calcaneus nor autopod length is rescued by either *Tbx4* or *Tbx5* transgenes (Fig. 1M,O,P,R and Fig. 2B). By contrast, the *Prx1-Pitx1* transgene can rescue formation of the patella bone and correct articulation of bones at the knee joint, the ratio of tibia/fibula diameter and formation of the calcaneus and autopod length (Fig. 1J-L and Fig. 2B). Significantly, we show that *Tbx5* or *Tbx4/5* chimeric transgenes are able to rescue the *Pitx1*$^{-/-}$ mutant outgrowth defect but all fail to rescue hindlimb morphologies to a similar degree as the *Tbx4* transgene. Together, these data are consistent with Pitx1 determining hindlimb morphology independently of Tbx4.

Pitx1 is necessary for hindlimb specific patterning of soft tissue independently of *Tbx4* activity

Although not previously described in detail, *Pitx1*$^{-/-}$ hindlimbs also have defects in muscle and tendon patterning. We used an anti-myosin antibody to examine muscle morphology in *Pitx1*$^{-/-}$ and *Prx1-Tbx4* rescued embryos. The size, shape and insertion sites of the muscles in the *Pitx1*$^{-/-}$ hindlimb are perturbed, consistent with *Pitx1* being necessary for correct morphogenesis and placement of the hindlimb muscles (Fig. 3C,H; supplementary material Fig. S3). The *Prx1-Pitx1* transgene is able to rescue the pattern of the hindlimb muscles in the *Pitx1* mutant (Fig. 3D,I; supplementary material Fig. S3) but *Prx1-Tbx4* and *Prx1-Tbx5* transgenes cannot (supplementary material Fig. S3). A good example of this difference in activity is provided by the extensor digitorum brevis (EDB), that comprises two muscles on the upper surface of the hindlimb autopod and the abductor digitorum quinti (AdQ) muscle lying on the lateral border of the hindlimb autopod (Fig. 3A,F). In the *Pitx1*$^{-/-}$ mutant, the EDB and AdQ muscles are absent (Fig. 3C,H). Formation of these muscles is rescued by the *Pitx1* transgene (Fig. 3D,I) but not by the *Tbx4* (Fig. 3E,J) or *Tbx5* (supplementary material Fig. S3) transgenes. These data demonstrate that the muscle defects observed in the *Pitx1*$^{-/-}$ mutant hindlimbs arise independently of the effects on *Tbx4* expression.

Muscles are anchored to bones via tendon attachments. We used the *Scx-GFP* reporter line (Pryce et al., 2007) to visualise the hindlimb tendon insertions. The peroneus longus (PL) muscle inserts at the base of the first metatarsal and cuneiform via a tendon that shares a common path across the lateral side of the calcaneus with the tendons of peroneus digitorum quatri (PDQa) and peroneus digitorum quinti (PDQi) muscles (Fig. 4A,E). The PL muscle and tendon are absent from the *Pitx1*$^{-/-}$ hindlimb (PL, Fig. 4A,C; data not shown). The PDQi/PDQa muscles located underneath the PL in the wild-type hindlimb and which normally insert into the fibula head, now occupy a superficial position in the *Pitx1*$^{-/-}$ mutant and are shifted from a lateral to a medial position (PDQi/PDQa, Fig. 4A,C). These defects might reflect the aberrant positioning and morphology of the fibula and calcaneus that prevents these tendons from following their normal path. The pattern of disrupted muscles and tendons is identical in *Pitx1*$^{-/-}$, *Pitx1*$^{-/-}$*;Prx1-Tbx4* (Fig. 4C,D) and *Pitx1*$^{-/-}$*;Prx1-Tbx5* (supplementary material Fig. S3G,H) hindlimbs. Together, these results are consistent with Pitx1 function being essential for correct hindlimb soft tissue patterning and, importantly, that the defects observed in *Pitx1*$^{-/-}$ hindlimbs cannot be rescued by *Tbx4*, indicating that they occur independently of the disruption of *Tbx4* expression.

Pitx1 acts between E13.5 and E14.5 to regulate growth rate of the metatarsal elements

One of the characteristic features of the hindlimb skeleton is the increased length of the metatarsal elements compared with the homologous metacarpal elements of the forelimb. The relative elongation of the metatarsals is partially responsible for the greater overall length of the hindlimb autopod compared with the forelimb autopod. The length of the hindlimb autopod is greatly reduced in the *Pitx1*$^{-/-}$ mouse and this defect is independent of *Tbx4* because in the *Pitx1*$^{-/-}$ mutant autopod length is not rescued by the *Tbx4* transgene (grey and yellow bars, Fig. 2B; supplementary material Fig. S4). The condensing metacarpal and metatarsal elements are first visible by Alcian Blue staining at E13.5 and initially are of similar size (Fig. 5A,B,U). By E17.5, the metatarsal elements are significantly longer than the metacarpals at the same stage, unlike the first phalangial elements of the forelimb and hindlimb, which show no statistical differences in size in this assay (Fig. 5Q,R; data not shown). Different scenarios can explain this size difference; the metacarpals might grow for a longer period of time than the metacarpals or the growth rate of the metatarsals might be accelerated compared with the metacarpals. Comparing the lengths of metacarpal and metatarsal elements between E13.5 and E17.5 revealed that significant differences in size are already

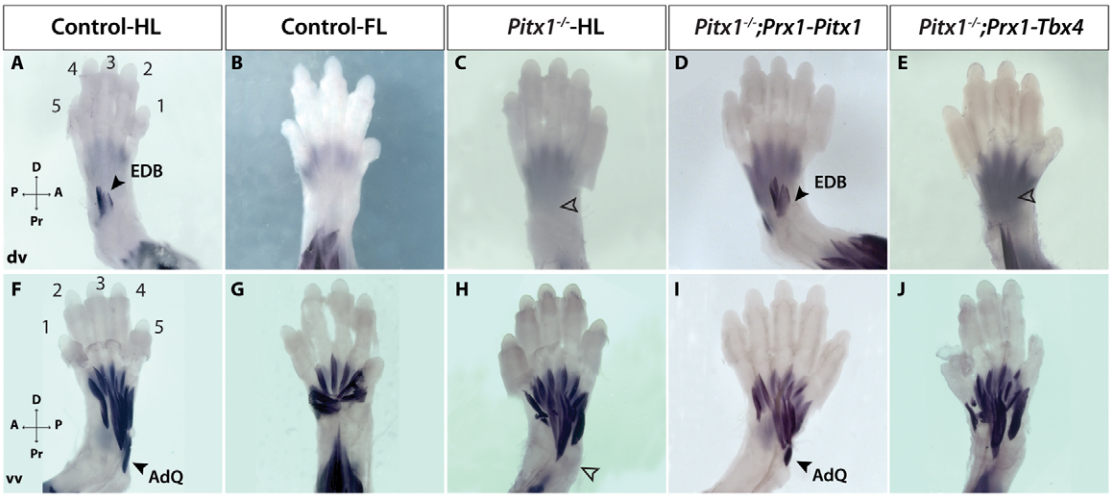

Fig. 3. Pitx1 is necessary for correct patterning of hindlimb muscles independently of *Tbx4* expression. Whole-mount immunohistochemistry of E15.5 mouse limbs using anti-muscle myosin antibody. (**A-E**) Dorsal views (dv) of the autopod region. The extensor digitorum brevis (EDB) in control hindlimb (HL) (A) does not have an equivalently located muscle in the forelimb (FL) (B). The EDB is absent from the *Pitx1*$^{-/-}$ hindlimb (open arrowhead; C). The formation of the EDB muscle is rescued by the *Prx1-Pitx1* transgene (D) but not by the *Prx1-Tbx4* transgene (E). (**F-J**) Ventral views (vv) of the autopod region. Similarly, the abductor quinti muscle (AdQ) is located in the ventral autopod of control hindlimbs (F) and has no equivalent in the forelimb (G). This muscle is absent from *Pitx1*$^{-/-}$ HL (H, open arrowhead). AdQ formation is rescued by the *Prx1-Pitx1* (I), but not by the *Prx1-Tbx4* (J) transgene.

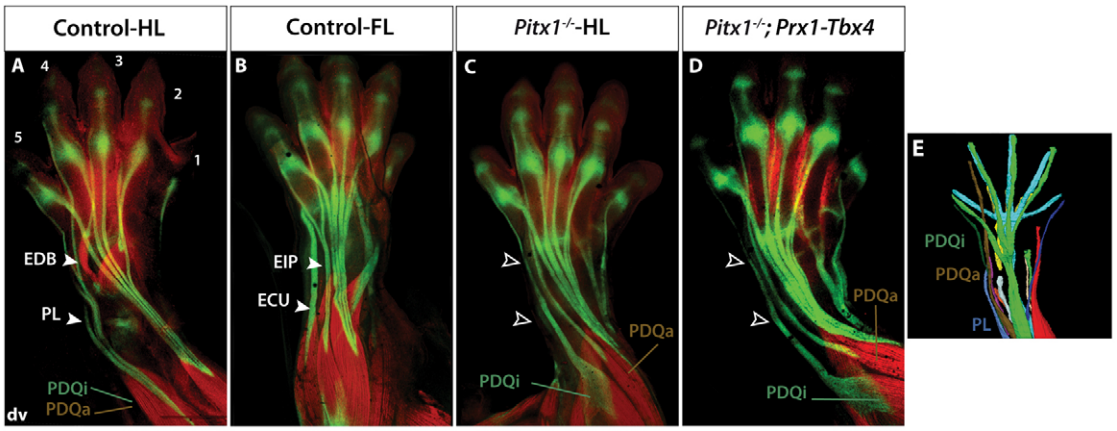

Fig. 4. Pitx1 is necessary for correct patterning of hindlimb tendons independently of *Tbx4* expression Confocal imaging (*z*-stacks) of whole-mount immunohistochemistry on E14.5 mouse limbs using the anti-muscle myosin antibody (red) and *Scx-GFP* reporter line (green) to reveal tendons. (**A**,**B**) The peroneus longus (PL) muscle and tendon and peroneus digiti quarti and quinti (PDQa, PDQi) are located in a lateral posterior position in the control HL (A). These muscles have no equivalent in the FL (B). (**C**,**D**) The PL is absent in the *Pitx1*$^{-/-}$ HL (open arrowhead), and the PDQa and PDQi muscle masses are shifted to a more anterolateral position (C). Similar defects are observed in the *Pitx1*$^{-/-}$; *Prx1-Tbx4* (D). (**E**) 3D rendering of datasets produced by optical projection tomography showing a ventral view of distal hindlimb tendons and muscles. ECU, extensor carpi ulnaris; EDB, extensor digitorum brevis; EIP, extensor indicis proprius.

detectable at E14.5 (Fig. 4U), ruling out the prolonged growth scenario. These results support a two-phase model for the growth of these elements: Phase 1 is an accelerated growth phase from E13.5 to E15.5 during which the metatarsal elements acquire their greater relative size and Phase 2 is a growth period from E15.5 when these homologous elements have similar growth rates. *Pitx1* is expressed in the surrounding of the metatarsal elements at the appropriate stages, consistent with Pitx1 having a role in regulating the growth rate of these structures (supplementary material Fig. S5A-D). In support of this model, this accelerated growth phase of the metatarsals is not observed in the *Pitx1*$^{-/-}$ mutant hindlimbs and these elements display a growth profile similar to metacarpals (Fig. 5U). In addition, metacarpal elements of *Prx1-Pitx1* transgenic forelimbs show an increase in their growth rate during this initial period of their development, demonstrating that ectopic *Pitx1* is sufficient to confer these growth dynamics on the homologous forelimb elements. Overall, these results suggest that Pitx1 shapes specific skeletal elements, such as the metatarsals, by increasing their growth rates during a fixed time period.

DISCUSSION

Pitx1 is the only known modulator of limb-type morphologies and, uniquely, has been shown in the mouse to be both required for the formation of hindlimb characteristics and to be sufficient to produce hindlimb-like structures when misexpressed in the forelimb. Using a gene replacement strategy in a *Pitx1*$^{-/-}$ mutant background, we have uncoupled the two major functions of Pitx1 during hindlimb development. First, this transcription factor has an input in controlling the ultimate size of the forming hindlimb elements by tuning *Tbx4* expression levels. Our results also demonstrate that Pitx1 acts as a regulator of hindlimb morphology independently of *Tbx4* and is crucial for proper shaping of hindlimb bone and soft tissues. Furthermore, we provide a first explanation of how Pitx1 sculpts the forming hindlimb skeleton through localised modulation of the growth rate of discrete elements, such as the metatarsals.

Pitx1 shapes hindlimb morphologies independently of *Tbx4* activity

Our data demonstrate that *Pitx1*$^{-/-}$ hindlimbs display a compound phenotype arising from two separable defects: a disruption of normal hindlimb outgrowth and a failure to determine some hindlimb morphological characteristics. Significantly, we show that *Tbx4*, *Tbx5* or *Tbx4/5* chimeric transgenes are able to rescue the *Pitx1*$^{-/-}$ mutant hindlimb outgrowth defect but all transgenes fail to rescue hindlimb morphologies. Together, these data demonstrate that Pitx1 determines hindlimb morphology independently of *Tbx4*. Our results differ from those recently published by Ouimette and colleagues (Ouimette et al., 2010) who have proposed that a unique Tbx4 repressor activity is the primary effector of hindlimb identity. This study used a similar transgene gene replacement strategy in the background of a *Pitx1* mutant. They did not, however, include a *Prx1-Pitx1* control rescue, as we have done, that provides a reference for the remaining experimental rescue transgenes. Crucially, they also did not provide controls for the efficacy of the *Tbx4* and *Tbx5* transgenes as we have done in our study. The divergence of the conclusions reached by our analyses derives from two main reasons. Their conclusions are based on an observed difference in the extent of rescue of the *Pitx1*$^{-/-}$ phenotype using a single *Tbx4* and single *Tbx5* transgene line for which they provide no control of activity. A simple explanation is that the *Tbx5* transgenic they have used is a ‘weaker’ line than their *Tbx4* transgenic and thus fails to rescue hindlimb outgrowth as effectively. Secondly, the criteria used by Ouimette et al. to assess the hindlimb rescue are flawed. Our study demonstrates that the morphological features used by Ouimette et al. can be rescued equally well by *Tbx5* and chimeric *Tbx4/5* transgenes and represent the structures that can form from the hindlimb-forming region in the absence of the influence of *Pitx1* (or *Tbx4*). The combination of these errors has led to a misinterpretation of what we show to be rescue of hindlimb outgrowth as a rescue of hindlimb morphology. In our study, we have carried out the essential systematic comparison of the relative activities of the *Prx1-Tbx4*, *Prx1-Tbx5* and chimeric

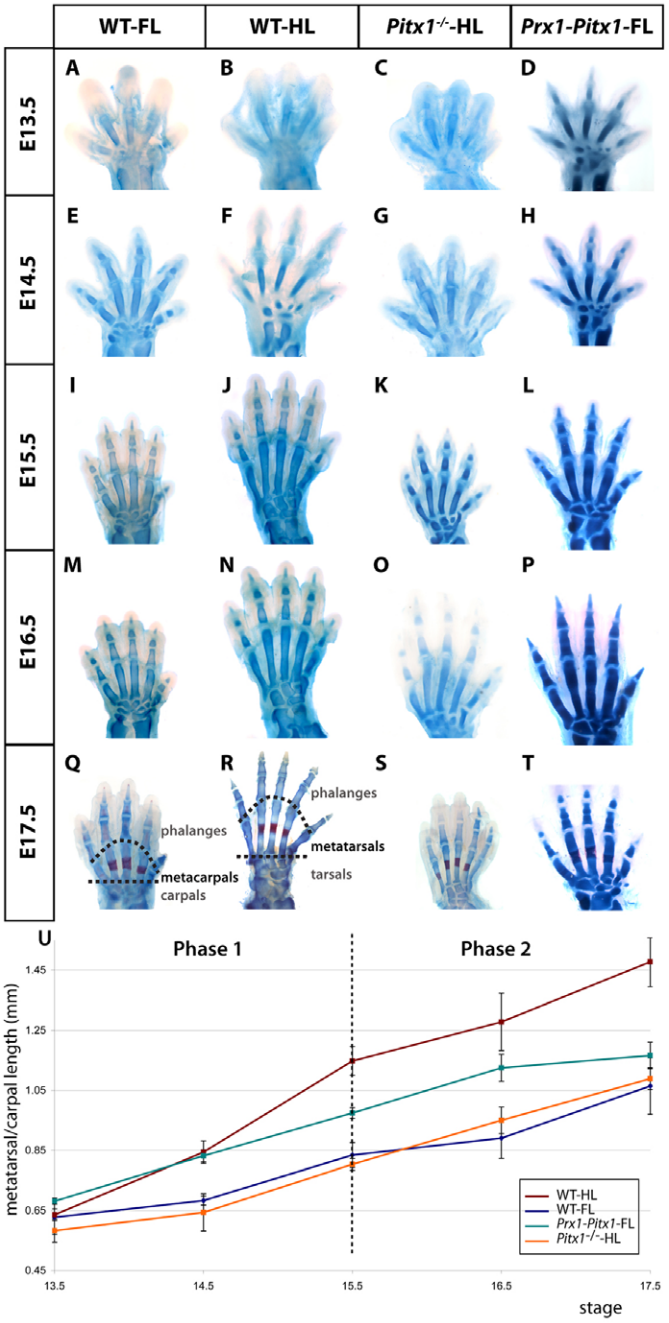

Fig. 5. Pitx1 influences metatarsal growth rate. (**A-T**) Skeletal preparations of autopods at stage E13.5 (A-D), E14.5 (E-H), E15.5 (I-L), E16.5 (M-P) and E17.5 (Q-T). Wild-type forelimb (WT-FL) (A,E,I,M,Q), wild-type hindlimb (WT-HL) (B,F,J,N,R), *Pitx1*$^{-/-}$ hindlimb (HL) (C,G,K,O,S) and *Prx1-Pitx1* forelimb (FL) (D,H,L,P,T). Measured metacarpal and metatarsal elements are outlined by dotted lines in Q and R. (**U**) Graph plotting the increase in length (mm) of the 3rd metatarsal or metacarpal element at different stages of development for WT-HL (brown), WT-FL (dark blue), *Prx1-Pitx1* FL (light green) and *Pitx1*$^{-/-}$ HL (orange). Two distinct phases of metatarsal growth are separated by the dashed line: in Phase 1, from E13.5-15.5, metatarsal growth rate is higher compared with metacarpals; in Phase 2, from E15.5-17.5, metatarsals and metacarpals growth rates are equivalent.

rescue transgenes and have demonstrated their abilities to rescue the *Tbx5* and *Tbx4* conditional knockout limb phenotypes. These controls show that our lines are expressing transgenes at levels sufficient for normal limb development. In our hands, Tbx4 acts as a transcriptional activator, which is consistent with both its established role to positively regulate *Fgf10* expression (Minguillon et al., 2005; Naiche and Papaioannou, 2007) and its ability to rescue limb formation in either *Tbx5* or *Tbx4* mutants (Minguillon et al., 2005; Minguillon et al., 2009) (data not shown).

Pitx1 controls initiation of hindlimb outgrowth during a brief, early phase through regulation of *Tbx4* expression, whereas Pitx1 determines hindlimb morphology in a broader time frame

The growth defect observed in the *Pitx1*$^{-/-}$ hindlimb demonstrates the importance of the positive transcriptional input of Pitx1 on *Tbx4* expression, which ensures that the appropriate levels of *Tbx4* necessary for the hindlimb to develop to its normal size are reached. *Tbx4* is required in a first phase during the earliest stages of hindlimb development for initiation of limb budding but does not contribute to further outgrowth of the hindlimb (Naiche and Papaioannou, 2007). In a second phase, *Tbx4* is required for correct patterning of the forming hindlimb soft tissues (Hasson et al., 2010) and is functionally dispensable for hindlimb development after E12.5 (Naiche and Papaioannou, 2007). It follows that the positive regulatory effect of *Pitx1* on *Tbx4*, and subsequently *Tbx4* on *Fgf10* expression, is temporally restricted to hindlimb bud initiation stages (Fig. 6). Disruption of the hindlimb initiation programme, therefore, ultimately affects the number and/or size of skeletal elements, possibly as a result of the specification of a smaller progenitor pool and/or failure to sufficiently expand this pool of progenitors in the emerging limb bud. By contrast, Pitx1 is acting in a broader time frame during hindlimb development, first by influencing the levels of *Tbx4* and, subsequently, in one specific example at least, by controlling skeleton morphology by regulating the growth rate of the metatarsal elements during later hindlimb development.

Pitx1 and hindlimb muscle patterning

Pitx1 is necessary for correct hindlimb-specific muscle pattern to form. Our results suggest that this happens in a *Tbx4*-independent manner as the hindlimb-specific pattern of soft tissue cannot be rescued by a *Tbx4* transgene. Previously, we have shown that *Tbx4* activity in connective tissue that surrounds the muscle and tendons is required for the forming hindlimb soft tissues to acquire their correct size, shape and insertion sites (Hasson et al., 2010). Significantly, the soft tissue phenotypes we have described in *Pitx1*$^{-/-}$ and conditional *Tbx4* mutants are distinct. We propose that *Pitx1* is required for normal levels of *Tbx4* during its first phase of activity, initiation of hindlimb budding. Subsequently, *Pitx1* might be dispensable for *Tbx4* expression in connective tissue. One other possibility is that the muscle defects observed in the *Pitx1*$^{-/-}$ background are secondary to the skeletal defects. Individual muscles are defined by their origin and insertion onto the skeleton. An abnormally formed skeleton can, therefore, lead to aberrant positioning of the muscles. Both the EDB and AdQ muscles that are affected in the *Pitx1* mutant share a common origin on the calcaneus bone. Our results do not distinguish whether the absence of these muscles is secondary to the loss of the bone insertion site or a primary defect in the nascent muscle bundles. Pitx1 might, therefore, act either autonomously in the emerging muscles masses or indirectly from connective tissue in the vicinity of these muscles to contribute to their ultimate pattern.

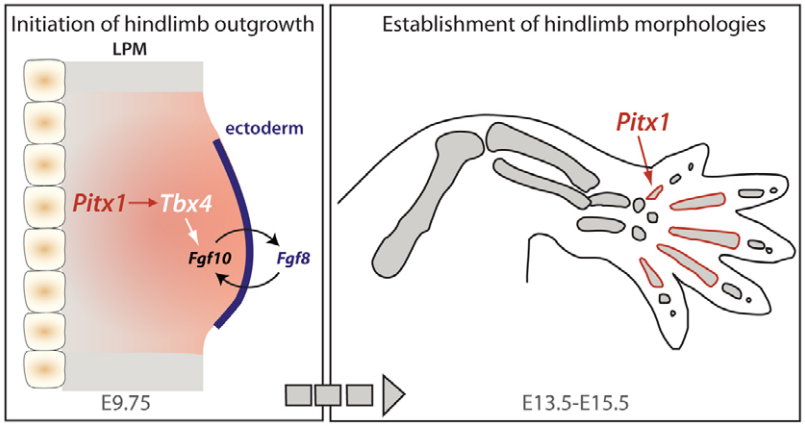

Fig. 6. *Pitx1* is necessary for normal initiation of hindlimb outgrowth through regulation of *Tbx4* expression levels and shapes hindlimb morphologies via targeted growth control. At pre-limb bud stages, *Pitx1* is required for normal initiation of hindlimb budding. *Pitx1* is necessary for normal expression levels of *Tbx4*, which helps to establish the Fgf10-Fgf8 positive feedback loop that is essential for limb outgrowth. Later, *Pitx1* expression is necessary for shaping of hindlimb morphologies independently of *Tbx4*. Between E13.5 and E15.5, expression of *Pitx1* surrounding the metatarsal elements leads to an increase in growth rate, resulting in their relative elongated shape compared with the homologous metacarpal elements. LPM, lateral plate mesoderm.

Genes required for formation of limb structures can be distinguished from genes that determine limb-type morphology

Forelimb and hindlimbs are serial homologous structures and the core regulatory networks employed during their development are thought to be acting equivalently. Nevertheless, the emerging morphologies of the forming limbs are distinct. There is an important distinction between factors strictly required for the emergence of limb structures and those factors required for shaping their final form. A good example of this distinction is provided by *Tbx5* and *Tbx4*. These factors are required for each limb element to form properly but not for the emergence of the limb-type morphology, as illustrated by their functional redundancy. *Tbx4* can replace *Tbx5* function in the forelimb (Minguillon et al., 2005; Minguillon et al., 2009) and, conversely, as we show here, *Tbx5* can substitute for hypomorphic *Tbx4* levels in *Pitx1*$^{-/-}$ mutant hindlimbs. Nevertheless, the duplication of the single ancestral gene to generate the *Tbx4* and *Tbx5* paralogous gene pair and the subsequent divergence in their expression patterns to either hindlimb or forelimb would have been instrumental in the forelimb and hindlimb being able to evolve more independently from one another.

By contrast, Pitx1 has an input in both formation of limb structures and the shaping of their ultimate morphology. The positive transcriptional input of Pitx1 ensures that the appropriate levels of *Tbx4* are reached for correct hindlimb size. In parallel, Pitx1 sculpts the forming hindlimb skeleton through localised modulation of the growth rate of discrete elements. Interestingly, our results show that expression of *Pitx1* in the forelimb is able to affect the metacarpal elements specifically, indicating that the homologous elements in the forelimb are competent to respond equivalently to this ectopic cue. The *Pitx1*$^{-/-}$ hindlimb phenotype does not represent an acquisition of forelimb characteristics but rather reflects a loss of some hindlimb characteristics. Neither the forelimb nor the hindlimb represents a default limb-type. Forelimb and hindlimb morphologies are derived states, in part, reflecting their divergent evolutionary histories and the influence of different selection pressures.

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The authors declare no competing financial interests.

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