Identification of limb-specific \textit{Lmx1b} auto-regulatory modules with Nail-patella syndrome pathogenicity

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\textit{LMX1B} haploinsufficiency causes Nail-patella syndrome (NPS; MIM 161200), characterized by nail dysplasia, absent/hypoplastic patellae, chronic kidney disease, and glaucoma. Accordingly in mice, \textit{Lmx1b} has been shown to play crucial roles in the development of the limb, kidney and eye. Although one functional allele of \textit{Lmx1b} appears adequate for development, \textit{Lmx1b} null mice display ventral-ventral distal limbs with abnormal kidney, eye and cerebellar development, more disruptive, but fully concordant with NPS. In \textit{Lmx1b} functional knockouts (KOs), \textit{Lmx1b} transcription in the limb is decreased nearly 6-fold, indicating autoregulation. Herein, we report on two conserved \textit{Lmx1b}-associated cis-regulatory modules (\textit{LARM1} and \textit{LARM2}) that are bound by Lmx1b, amplify \textit{Lmx1b} expression with unique spatial modularity in the limb, and are necessary for Lmx1b-mediated limb dorsalization. These enhancers, being conserved across vertebrates (including coelacanth, but not other fish species), and required for normal locomotion, provide a unique opportunity to study the role of dorsalization in the fin to limb transition. We also report on two NPS patient families with normal \textit{LMX1B} coding sequence, but with loss-of-function variations in the \textit{LARM1/2} region, stressing the role of regulatory modules in disease pathogenesis.

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The LIM homeodomain transcription factor Lmx1b is responsible for limb dorsalization. In the limb, Lmx1b is induced by Wnt7a from the dorsal ectoderm, and its expression is restricted to the dorsal mesoderm. Loss of Lmx1b function in mice results in loss of dorsal autopod (hand/foot) and zeugopod (forearm/leg) patterning: the autopods have a symmetrical ventral-ventral phenotype with dorsal footpads, loss of dorsal hair follicles, absence of nails, and a symmetrical ventral pattern of muscles, tendons and ligaments. Besides the limb, mice lacking functional Lmx1b exhibit abnormal eye, cerebral, and kidney development which accounts for the perinatal lethality. In contrast to mice, single allele variations in humans that disrupt LMX1B function cause Nail-patella syndrome (NPS; MIM 161200). This autosomal dominant condition is characterized by nail dysplasia, absent or hypoplastic patellae, bone fragility, premature osteoarthritis, chronic kidney disease, and ocular anomalies. Evaluation of the variety of human LMX1B mutations indicate that NPS is due to haploinsufficiency. Thus, a sub-threshold level of LMX1B is responsible for the syndromic features and incomplete limb dorsalization. In the murine model, homozygous KO mice exhibit a more dramatic phenotype than the human condition, with ventral-ventral distal limbs suggesting a threshold-mediated effect on limb dorsalization. In the absence of Lmx1b function, transcription of the Lmx1b mRNA is decreased nearly sixfold in developing (e12.5) mouse limbs suggesting that one function of Lmx1b is the auto-amplification of its own expression (Supplementary Table 1). Lmx1b-targeted chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-seq) during limb dorsalization (e12.5) identified two highly conserved Lmx1b-bound cis-regulatory modules (CRMs) 60 kb upstream of the Lmx1b gene. CRMs are DNA sequences enriched in transcription factor binding sites that regulate associated genes in a time- and tissue-specific manner. Lmx1b-binding to CRMs upstream of its own coding sequence provides a mechanism by which Lmx1b could auto-amplify its own expression. In this study, we have characterized and functionally validated these two Lmx1b-associated regulatory modules that we term LARM1 and LARM2. We show that they are highly conserved across vertebrates including coelacanth, but not other fish species. The activity of these two LARM sequences overlaps the expression pattern of Lmx1b in the dorsal limb mesoderm when assessed either together or individually using both chick and mouse enhancer assays. Removal of the LARM region with CRISPR-Cas9 results in a limb phenotype similar to that of animals lacking functional Lmx1b, with marked reduction in Lmx1b expression and loss of limb dorsoventral asymmetry, but without any other Lmx1b-related organ system affected. These data establish LARM1 and LARM2 as limb-specific Lmx1b enhancers necessary for amplifying the level of Lmx1b expression in the limbs. Interestingly, about 10% of patients with the NPS phenotype lack a variation in the Lmx1b coding sequence. We investigated two NPS patient families that lack coding sequence changes but instead have LARM variations that disrupt human LARM activity, highlighting the important role of cis-regulatory modules in development and disease pathogenesis.

Results

Lmx1b-associated regulatory modules are activated by LMX1B.

We recently identified two Lmx1b-bound CRMs, LARM1 and LARM2, 60 and 66 kb upstream of the mouse Lmx1b gene, respectively (Fig. 1a), that are associated with active chromatin marks (H3K27me3). For a given cell, histone acetylation (H3K27ac) and methylation (H3K27me3) are mutually exclusive; their coexistence indicates tissue heterogeneity. This pattern is consistent with LARM2 being only accessible and active in the dorsal limb compartment.

Using GFP reporter constructs in chicken electroporation bioassays, we found that both LARM1 and LARM2 demonstrate enhancer activity within the dorsal limb mesoderm coincident with the Lmx1b expression domain (Fig. 2a, i). Conservation analysis using multi-species alignment (Fig. 1b) subdivided LARM1 into two conserved regions, a 5′ element containing one conserved potential Lmx1b binding site and a 3′ element with three conserved binding sites (based on the reported TMATWA binding motif) (Fig. 2a). Surprisingly, the isolated 5′ LARM1 element did not show reporter activity, whereas the isolated 3′ LARM1 element showed strong activity in the limb mesoderm but with no dorsal bias (Fig. 2c). Interestingly, the restriction of LARM1 activity to the dorsal mesoderm requires the Lmx1b binding site within the 5′ element as site-directed mutations expanded enhancer activity into the ventral mesoderm (Fig. 2d). To ensure that a new permissive binding site had not been added, we generated two additional mutants of this Lmx1b binding site in the 5′ element of LARM1 and both also showed expanded enhancer activity into the ventral mesoderm (Supplementary Fig. 1). In contrast, mutation of any of the three predicted Lmx1b binding sites in the 3′ element resulted in markedly reduced yet still dorsally restricted LARM1 activity (Fig. 2d–e). These findings are counterintuitive since Lmx1b is only expressed in the dorsal limb mesoderm. A possible interpretation is that the putative Lmx1b binding site within the 5′ LARM1 element (TTATTA) can bind other transcription factors or corepressors that silence the 3′ LARM1 enhancer activity or promote chromatin conformation that limits enhancer-promoter interaction. In the dorsal mesoderm, Lmx1b would compete for this binding site, counteracting the silencer function of the 5′ LARM1 element, and drive enhancer activity. In support of this view, human LMX1B activates this enhancer when expressed together ectopically in the ventral limb bud mesoderm (Fig. 2j). Collectively, our data indicate that LARM1 is composed of a 3′ enhancer (LARM1e) and a 5′ silencer (LARM1s) that blocks ventral activity, thereby restricting its function to the dorsal limb.

In contrast, LARM2 is composed of a single conserved element containing two highly conserved Lmx1b binding sites (Fig. 2g). The dorsally restricted activity of LARM2 is abolished by disruption of either binding site (Fig. 2h, i) suggesting that LARM2 is a positive Lmx1b enhancer whose activity depends on Lmx1b. Furthermore, the observation that LARM2 reporter activity is activated in the ventral limb mesoderm by ectopically expressing human LMX1B fully corroborates this conclusion (Fig. 2j). These results, together with the pattern of chromatin marks in the LARM2 enhancer (Fig. 1b), suggest that Lmx1b plays a role in chromatin/LARM2 activation within the dorsal compartment.

Thus, our results show that both LARM1 and LARM2 are bound by Lmx1b, display dorsal restricted activity in limb buds, require Lmx1b binding sites for activity, and are activated by human LMX1B when co-expressed in the ventral mesoderm. This, together with published capture C experiments showing that the LARM region physically interacts with the Lmx1b promoter (Supplementary Fig. 2), supports the concept that LARM1 and LARM2 are bona fide Lmx1b autoregulatory enhancers.

We note that the Lmx1b locus includes a long non-coding RNA (C13002120) (https://doi.org/10.1371/journal.pone.0028358; http://www.biomedcentral.com/1471-213X/11/47) that is transcribed...
from the opposite strand of Lmx1b using the same bidirectional promoter20,21. This lncRNA transcript shows the same dorsal pattern of expression as Lmx1b during limb development and it is very likely that it shares in the LARM cis-regulation.

Interestingly, apDV, one of the enhancers of Apterous (ap), the Drosophila homologue of Lmx1b, is maintained by a positive autoregulatory loop, albeit indirectly through the ap targets vestigial and scollop (Vg/Sd)22. This suggests that positive autoregulation of Lmx1b is a conserved mechanism. In Drosophila, the apDV enhancer can only be active after ap induction by another early enhancer (apE). In the murine model, our results indicate that the LARM enhancers are not necessary for the initial activation of Lmx1b in the limb, but rather for its subsequent amplification above threshold levels. We also note the presence of another potential CRM 10 kb upstream of LARM2 (Fig. 1a and Supplementary Fig. 2, asterisk) as a candidate for early Lmx1b induction that does not appear to require Lmx1b binding (i.e., it was not bound by Lmx1b in a Lmx1b-ChIP-seq at e12.5)8. Certainly, the presence of additional enhancers in the Lmx1b locus merits further investigation.

LARM activity is required for limb-specific Lmx1b amplification. To determine their functional role in Lmx1b regulation, we deleted the LARM region by CRISPR-Cas9. Mice homozygous for a 7.6 kb deletion encompassing both LARM1 and LARM2 (ΔLARM1/2) exhibit a limb phenotype similar to that observed in the absence of functional Lmx1b8 displaying a loss of limb dorsalization, i.e., distal ventral-ventral limbs that involve the skeleton, muscles and integument (Figs. 3 and Supplementary Fig. 3). Micro-computed tomography (microCT) demonstrates biventral distal skeletal elements (Fig. 3b–f' for the hindlimb and Supplementary Fig. 3 for the forelimb) with dorsoventral symmetrical distal phalanges (Fig. 3c–e' and Supplementary Fig. 3), sesamoid bones (Fig. 3d–d and Supplementary Fig. 3) and tali (Fig. 3e–e'). In addition, the patella, the dorsal most structure of the knee, is absent (also a notable feature in Lmx1b KO mice and NPS patients) (Fig. 3f–f'). These skeletal abnormalities are accompanied by corresponding muscular abnormalities (Fig. 3g–g').

In addition to the loss of limb dorsalization, the development of the cerebellum, kidney, and eye is also affected3 in Lmx1b KO mice, and mutant mice die shortly after birth. However, homozygous ΔLARM1/2 mice are viable, and the organs affected in Lmx1b KO mice appear normal in the absence of the LARM region, indicating the limb-specific function of these two enhancers (Supplementary Fig. 4). The analysis of Lmx1b expression in ΔLARM1/2 embryos by whole mount in situ hybridization shows a normal pattern except in the limb where it was below detection limits (Fig. 3h). Analysis of limb Lmx1b RNA by RT-qPCR at e12.5 demonstrates a significant decrease of 60% in the steady state level of Lmx1b mRNA compared to normal mice (Fig. 3i). As mentioned above, the persistent expression of Lmx1b, albeit at a lower level in the ΔLARM1/2 mice, suggests that additional CRMs may be involved in the induction of Lmx1b, while LARM1/2 amplify Lmx1b to levels adequate to accomplish dorsalization. Indeed a potential CRM is present 10 kb upstream of LARM2 (Fig. 1a and Supplementary Fig. 2, asterisk), does not appear to bind Lmx1b (it was not identified by Lmx1b ChIP-seq analysis)8, but overlaps with several chromatin-associated marks indicative of active regulation (Supplementary Fig. 2), and is worthy of further investigation. Collectively, our results establish

![Image](https://example.com/image.png)
LARM1/2 as limb-specific Lmx1b autoregulatory CRMs that are necessary for normal Lmx1b transcription levels.

**Human LARM1 and LARM2 activity and role in NPS.** LARM1/2 are conserved in humans, including the LMX1B binding sites (Figs. 1 and 2). We isolated the hLARM sequences and demonstrated dorsally restricted activity in the chick bioassay, either isolated or together (Fig. 4a). We also evaluated the hLARM sequences in transgenic mice at e12.5 (Fig. 4b). The LARM transgenes displayed a limb-restricted and dorsally accentuated activity. LARM1 exhibited accentuated activity in the dorsal limb mesoderm, but weaker activity was also evident in the distal ventral aspect. LARM2 was tightly restricted to the dorsal limb mesoderm but also lacked activity in the fifth digital ray (Fig. 4b, arrow). The transgene including the entire LARM region had
dorsally restricted expression with the exception of a small ventral patch in the presumptive carpal/tarsal region (Fig. 4b, arrowhead), suggesting a cooperative implementation of LARM1 and LARM2 activities for the refinement of dorsal restriction/enhancement.

We also explored the LARM region in 11 unrelated patients affected with NPS lacking sequence or copy number variation of the LMX1B coding region. Five of these patients were reported in a recent study9. In one proband (IV-7), we identified a 4.5 kb heterozygous deletion (Decipher database ID#433715) encompassing all of LARM2 and an adjacent downstream region (Family 53 from Ghoumid et al.)9 (Fig. 5a, b). We found that the LARM deletion segregates in one affected cousin (IV-1) and inferred from that result that two other affected individuals are obligate carriers (III-1 and III-4). Remarkably, individuals from this family exhibit nail dysplasia and patella hypoplasia, without ocular or renal involvement (Fig. 5c–g and k–n individual IV-7 and Fig. 5h–j individual IV-1). The nail defects were predominant on the first and second rays (kolonichya affecting thumbs and index fingers, longitudinal striations affecting hallucles) for the two individuals described, but the 5th rays were also mildly affected (nail hypoplasia of 5th fingers and toes) (Fig. 5c–j). The phenotype in this family is limb-restricted consistent with loss of enhancer activity within the LARM region. To investigate the phenotypic effects of the LARM deletion in this family, we generated by CRISPR-Cas9 a mouse model that replicated the 4.5 kb deletion carried by proband IV-7. Mice lacking this LARM variant had reduced activity (Fig. 5p). However, a construct containing only the rare SNV within LARM2 did not appreciably interfere with the dorsal expression of LMX1B.

Discussion

In this report, we characterize two conserved Lmx1b-associated cis-regulatory modules (LARM1 and LARM2) that are bound by Lmx1b and required to amplify Lmx1b expression in the limb to levels sufficient to accomplish limb dorsalization. Thus, LARM1 and LARM2 are two limb-specific Lmx1b enhancers that display remarkable modular spatial activity and that are required for establishing the correct dorsoventral pattern across the anterior-posterior axis.

Consistent with being limb-specific enhancers, mice in which the LARM region has been removed do not develop other Lmx1b-associated abnormalities that might jeopardize survival, thereby offering an extraordinary opportunity to study the functional capacities of ventral-ventral limbs. Indeed, the limbs of ΔLARM1/2 mice are insufficient for locomotion. ΔLARM1/2 mice cannot walk, but rather use an undulating or irregular wiggling motion because their limbs are unable to lift their bodies to move them forward. This stresses the notion that fins capable of supporting the body weight, such as those observed in Tiktaalik, must have been an initial step in the transition to tetrapods23. Considering that animal forms likely evolved by altering the regulation of key developmental genes, modification of the Lmx1b landscape may have been a critical step in the acquisition of dorsoventral polarized fins capable of lifting and moving the body, a hypothesis that deserves further investigation. In contrast, the partial (or modular) dorsoventral alterations exhibited by ΔLARM1 and ΔLARM2 homozygous mice do not appreciably interfere with
locomotion, although abnormal gait has been occasionally observed.

We also show that disruption of these enhancers can cause human pathology since loss-of-function variations in the LARM region are responsible for a limb-specific form of human NPS. This limited form of NPS is not associated with the typical risk of chronic kidney disease or glaucoma. Moreover, there is no protein-coding variation. Recognition that disruption of the LARM region can cause this limited form of NPS provides these patients with a more accurate assessment of their condition.

The NPS phenotype is attributed to haploinsufficiency, i.e., reduced levels of LMX1B due to the loss of one allele. Our studies further characterize the pathogenicity of NPS to reduced levels of LMX1B. In one NPS family, a single allelic deletion of LARM2...
yielding limb features diagnostic of NPS (incomplete limb dorsali-
zation) indicating that LMX1B protein levels are below the nor-
mal patterning threshold. In another family, homozygosity of a
functionally impaired LARM2 allele also yields limb features
diagnostic of NPS. In both of these families, the remaining
LARM1 enhancer, which demonstrates clear activity in transgenic
mice and chicken bioassays, appear able to support LMX1B
amplification to partially dorsalize the posterior limb and avert a
more severe ventral-ventral phenotype. During the final sub-
mission of this manuscript an additional NPS family with a
confirmed deletion that removed both LARM1 and LARM2 in
one allele was identified. Further mapping and studies are
underway. Together our results point to the contribution of both
allelic sets of the LARM enhancers to get a fully functional dose
of LMX1B across the anterior-posterior axis.
Congenital limb anomalies are relatively common24,25 with
syndromic forms associated with more than a hundred genes.
The association of multiple affected organs (developmental pleio-
trropy) provides a clue to the affected gene and permits a high
diagnostic yield. However, more than half of limb anomalies are
isolated without other malformations, and the diagnostic yield
of genetic evaluation remains low in these cases due to, at least in
part, an emphasis on evaluating coding sequences. During mor-
phogenesis, tissue-specific CRMs cause developmental pleiotropy
by regulating genes in key developmental pathways in precise
temporal and spatial patterns. Thus, tissue-specific CRMs are
potential candidates to explain isolated limb anomalies. Our
findings, as well as others linking limb-specific CRMs to limb
anomalies26–29, support this concept. Characterization of CRM-
disease associations represents a forthcoming opportunity in
clinical genetics, not only for limb anomalies, but also for other
isolated malformations.

Methods
Animal procedures. All animal procedures were reviewed and approved by
the Loma Linda University Institutional Animal Care Use Committee (IACUC) or by
the Bioethics Committee of the University of Cantabria and performed according
to the EU regulations, animal welfare and 3R principles. Representative images are
showed, but in all animal analyses performed, at least two independent specimens,
and in most cases three or more, were used to confirm the morphologic pattern.

Patients. We obtained informed written consent from all participants for genetic
analyses. Analyses were performed on a diagnosis basis in the University hospital
of Lille, following the bioethics rules of French law. The study was reviewed by the
Institutional Ethics Committee of the University of Lille and was found to be in
accordance with the criteria set by the Declaration of Helsinki. No identifiable
images of human participants are used.

Functional enhancer validation in chicken bioassays. We used a thymidine
kinase (tk) promoter-driven GFP reporter (kind gift of Masanori Uchikawa)30 to
generate enhancer constructs. Functional analyses of the LARM1 and LARM2
constructs were performed by electroporation into presumptive limb mesoderm of
Hamburger and Hamilton stage (HH) 14 chicken embryos. Co-electroporation of a
β-actin promoter-driven RFP construct (pCAGGS-RFP, kind gift from Cheryl
Tickle)31 was used to determine transfection efficiency. Electroporation was per-
formed using the CUT21 electroporation station (Protech International, Boerne,
TX). Embryos were incubated for 48 h before harvesting for visualization of GFP
activity and digital image acquisition (Sony DKC-5000) into Adobe Photoshop
(version 6.0, acquisition; version 2020, compilation).

To demonstrate that LMX1B could induce construct activity, pCDNA3.1-hLMX1B
(kind gift from Roy Moreno)32 was co-electroporated with either ptk-LARM1-GFP or
ptk-LARM2-GFP into the ventral mesoderm of stage HH23 chicken limb buds.
Cloning and site-directed mutagenesis. Primers used for the isolation of enhancer sequences from genomic DNA are listed in Supplementary Data 1. Disruption of the Lmxb1 binding sites was performed using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) following manufacturer recommendations and confirmed by Sanger sequencing. Briefly, nucleotides were modified to disrupt the binding site with a change of at least 3 nucleotides, not add another binding site, and add a restriction enzyme site for evaluation of successful mutagenesis. All potential binding site changes were evaluated by AliBaba2.132 and/or TRANSFAC® Cloning and site-directed mutagenesis for evaluation of successful mutagenesis. All potential binding site changes were evaluated by AliBaba2.132 and/or TRANSFAC® prior to construction to ensure that no new binding sites present in the limb were introduced.

In vivo transgenic reporter assays. Lmx1b-associated regulatory modules were isolated from human genomic DNA with the primers listed Supplementary Data 1 and cloned into the hsp68-LacZ kindly provided by Dr. Ahituv34. The constructs were used to generate transgenic embryos (Cyagen Transgenic service, Santa Clara, California). The embryos were harvested at e12.5 and processed for LacZ activity.

Analyses of published data. Limb ChIP-seq data were obtained from the Gene Expression Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) under the accession numbers GSE84064 for Lmx1b1, GSE2413 for H3K27Ac, GSE13845 for p300, and GSE42237 for both H3K27me3 and H3K4me1. RNA Pol II and Med12 ChIP-seq data were available from Berlivet and coworkers13. Previously published data containing the genomic coordinates of interest were uploaded to the UCSC genome browser and converted to the mouse build mm10 using the liftover tool. Capture-C data were mined from previous published work deposited in the GEO database under accession number GSM2251518.

CRISPR-Cas9 mediated enhancer knockout mice generation. The knockout mouse strain for the LARM region (LARM/LARM) was generated with the use of CRISPR-Cas9. Single guides RNAs (sgRNA) flanking the LARM locus listed in Supplementary Data 1 were designed using breaking-Cas35. The sgRNAs were generated (Sigma-Aldrich) and cleavage efficiency tested by Sigma-Aldrich using their cel-1 assay in mouse neuroblastema (N2a) cells with the primer pairs listed in Supplementary Data 1. The sgRNAs were delivered to the hearts of 1-day-old embryos by electroporation. The treated embryos were placed in donor CD1 pseudopregnant females (mated with vasectomized CD1 males to induce pseudopregnancy). Genotyping of founder (F0) mice for the identification of the desired deletion was performed with the use of the Phusion high fidelity polymerase (Thermo Scientific) with the primers listed in Supplementary Data 1. A founder female B6CBA was bred to

Fig. 5 Clinical features of LARM loss-of-function. a Pedigree of a family with a LARM deletion. b The 4.5 kb region deleted removes all of LARM. c-g, k-n Phenotypic images of individual IV-7. c-d Kollonychia of thumb and 2nd finger. e-f Triangular lunulae of 3rd and 4th fingers. g Nail dysplasia of the hallux showing longitudinal striations. h-j Phenotypic images of individual IV-1. h Kollonychia of thumb. i Hypoplastic nails, ungual dysplasia of 2nd finger. j Ungual dysplasia of right foot predominating on 1st and 5th toes. k-n Knee X-rays showing bilateral hypoplasia of the patella. o Schematic of the patient’s chromosome 9 showing large segments of the chromosome with loss of heterozygosity (LOH), i.e., homozygosity, when comparing the allele frequencies to the Log R ratio of the alleles. One of the homozygous regions includes the LARM-LMX1B locus. The homozygous hLARM2 sequence showing the five single nucleotide variations (SNVs). The asterisk indicates the rare (0.08%) sequence in the cohort. p Using site-directed mutagenesis, we generated a human LARM2 construct containing the patient´ s 5 SNVs; following electroporation into embryonic chick wings, the patient-LARM2 sequence showed markedly reduced activity (n = 6; compare with the activity of the common hLARM2 sequence in Fig. 4a). Interestingly, mutation of only the single SNV within the conserved LARM2 region did not alter LARM2 activity.
C57/B6 for the generation of the LARM1/2 knockout mice strain after performing Sanger sequencing of the PCR amplicons that confirmed a 7,564 bp deletion encompassing the LARM locus (Chr2: 33555354-33563008; Mouse July 2007(NCBI/mm9) assembly). The genotyping strategy included Sanger sequencing of the PCR amplicons for the off-target regions providing the highest score for each of the sgRNAs according to Breaking-Cas with the use of the primers listed in Supplementary Data 1. The mutant lines were generated by electroporation using the Alt-R CRISPR-Cas9 System from IDT (https://eu.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system) and the NEPA21 electroporator and the RT-qPCR was carried out on an Applied Biosystems StepOnePlus using NZYSpeedy qPCR Green Master Mix, ROX plus (NZYTech). The primers used to amplify LARM1/2 homozygous embryos were designed using CHOPCHOP (https://chopchop.cbu.uib.no/) and are listed in Supplementary Data 1. The mutant lines were generated by electroporation using the Alt-R CRISPR-Cas9 System from IDT (https://eu.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system) and the NEPA21 electroporator and the RT-qPCR was carried out on an Applied Biosystems StepOnePlus using NZYSpeedy qPCR Green Master Mix, ROX plus (NZYTech). The primers used to amplify Lmx1b were forward (Fwd), GAGCAAAGATGAAGAAGCTGGC37, and reverse (Rev), GGCCACGA37. Three-week-old mouse limbs were scanned with Skyscan1172 at 40 kV, 95% ethanol. Alizarin red and alcian blue skeletal staining was performed following standard procedures using the NRecon reconstruction software (Ver 1.6.10.2) and compiled with the CTVox version 3.3.1volume rendering software.

**Histology.** Animals were subjected to intravascular perfusion of 4% PFA with the use of a peristaltic bomb. Gross morphologic and histologic analyses were performed on the limbs, skull, brainstem, kidneys, and eyes. The soft tissues (brain, kidneys and eyes) were fixed in 10% phosphate-buffered formalin, while the limbs were decalcified then post-fixed with 10% phosphate-buffered formalin. The tissues were paraffin-embedded following standard procedures and stained with hematoxylin and eosin.

**RT-qPCR.** The hindlimbs from e12.5 wild type and LARM1/2 homozygous embryos were dissected out in cold RNase-free 1X phosphate-buffered saline (PBS) pH 7.4. Total RNA was extracted with RNeasy Plus Mini Kit (Qiagen) and 500 ng of total RNA was reverse transcribed to produce first-strand cDNA with 5xScipTiM cDNA Synthesis kit (Bio-Rad) using standard conditions. RT-qPCR was carried out on Applied Biosystems StepOnePlus using NZYSpeedy qPCR Green Master Mix, ROX plus (NZYTech). The primers used to amplify Lmx1b were forward (Fwd), GAGCAAAGATGAAGAAGCTGGC37, and reverse (Rev), GGCCACGA37. Relative transcript levels were normalized to GAPDH (Fwd, TGCAGTGGCAAAGTGGAGAT; Rev, ACTGTGCCGTTGAATTTGCC). Three-four biological replicates were analyzed for each genotype, with 3 technical replicates per sample. The expression levels of mutant samples were calculated relative to wild-type controls (average set to 1). Significance of differences were determined using the two-tailed, unpaired t-test and reported with standard deviation error bars.

**In situ hybridization.** Whole-mount in situ hybridization was performed following standard procedures39 using digoxigenin-labeled antisense RNA probes for mouse Lmx1b5 and Human LMX1B5. Briefly, embryos were harvested in cold RNase-free 1x PBS (pH 7.4), fixed overnight cold (4 °C) MEMFA (100 mM MOPS (pH 7.4), 2 mM EGTA, 1 mM MgSO4, 3.7% (v/v) formaldehyde) and post-fixed overnight in 90% methanol at −20 °C. The embryos are then rehydrated in graded alcohols/PBS + 0.1% Tween, treated with protease K (10 mg/ml) for 20 minutes, rinsed in 0.1 M triethanolamine (TEA), and acetylated.
RNA Pol II and Med12 ChIP-Seq datasets are available as Supplementary datasets 1 and 2. The embryos were imaged using Leica M20 dissecting microscope with attached Sony DKC-5000 camera into Adobe Photoshop (version 6.0, acquisition; version 2020, compilation). To generate the chicken Lmx1b probe we isolated a 729 bp fragment from chicken cdNA that spans 5 introns (exons 2-7) of the Lmx1b gene using the following primer pairs: FWD: 5′ GGATGTCGTTCTGTGATGAG 3′, REV: 5′ GATGTCATCTCTCTCTGATCG 3′. The isolated fragment was ligated into pCRII-TOPO with dual Sp6 and T7 promoters for in vitro transcription (ThermoFisher Scientific) and sequence verified by Sanger sequencing.

LARM screening in NPS patients

DNA from patients was extracted from blood according to standard methods. LARM1 and LARM2 were sequenced on an ABI Prism 3730XL Genetic Analyzer. Additional embryos from the human LMX1B gene array deletion described in this study has been reported in the Decipher database under the accession number GSM2251518. Source data are provided with this paper. The data generated in Fig.3I of this study are provided as supplementary data. The colorized embryos were imaged by chick Lmx1 during vertebrate limb development. Published limb ChIP-seq data are available under the GEO database accession numbers: GSE84064 for Lmx1b, ID#433715 (https://www.deciphergenomics.org/). Publically available Lmx1b gene array data from patients involved of LMX1B in human Nail patella syndrome.

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Author contributions
E.H., F.P., M.A.R. and K.C.O conceived and designed the project. E.H., C.U.P, C.D.S., L.A.I, A.L.G. and A.N. acquired and analyzed murine and human LARM1/2 characterization studies in chickens. E.H., C.U.P, A.L.G. and K.C.O acquired and analyzed human LARM1/2 studies in transgenic mice. E.H., S.L., K.C.O and M.A.R generated and analyzed the CRISPR-cas9 knockout model mice. F.P. and S.M.H. supervised the acquisition and analysis of the human Nail-Patella Syndrome (NPS) data. F.E., A.-S.J., F.F., J.-M.G. and C.F. acquired and analyzed human NPS data. E.H., F.P., K.C.O. and M.A.R. drafted the manuscript. All authors were involved in editing/revising the manuscript.

Competing interests
The authors declare no competing interests.

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