Supporting Information

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SI Materials and Methods

Mice. The *Hm* mutant (The Jackson Laboratory) was backcrossed to C57BL/6JJcl, and the *Hm* allele is currently maintained in the C57BL/6JJcl background at the NIG. C57BL/6JJcl was purchased from Clear Japan and used as the wild-type control mouse in all experiments except for those depicted in Fig. S3*C*, in which we used MCH (ICR). For the CRISPR/Cas9 system, eggs of C57BL/6JJcl were fertilized in vitro with sperm from a homozygote of *Hm*. C57BL/6JJcl, MCH (ICR), and (C57BL/6JJcl × DBA/2J) F1 mice were used for transgenic reporter assays. To transmit the *Hm-inv* allele to the next generation, we employed in vitro fertilization with sperm from a G0 mouse. Three wild mouse-derived inbred strains—KJR/Ms, BLG/Ms and MSM/Ms—were established and maintained at NIG. An *Shh* coding sequence KO mouse strain (*Shh* KO) (a gift from P. Beachy, Stanford University, Stanford, CA), which was originally made in the 129/SV background, was backcrossed to C57BL/6JJcl at NIG. Animal experiments in this study were approved by the Animal Care and Use Committee of NIG.

X-Ray Micro-CT Analysis. Mouse autopods were scanned using X-ray micro-CT (ScanXmate-E090S105) (Comscantechno). Instrument settings for data acquisition are listed in Dataset S4. Before scanning, P0 autopods were soaked in contrast agent, a 1:3 mixture of Lugol's solution and deionized distilled water, for 24 h. 3D tomographic images were obtained using the OsiriX program (www.osirix-viewer.com) for P0 autopod and Tri/3D-BON (Ratoc System Engineering) for bones of 5-wk-old mice.

Whole-Mount in Situ Hybridization. In situ hybridization was carried out using the standard method (1). We linearized template vectors and synthesized riboprobes using a DIG RNA labeling mix (Roche) and T7 RNA polymerase (Promega). Mouse embryos were fixed with 4% paraformaldehyde at 4 °C overnight. After digestion with proteinase K (Invitrogen) and fixation in 4% paraformaldehyde containing 0.2% glutaraldehyde, embryos were hybridized in 50% formamide containing 5xSSC (pH 5.0) and 1% SDS. Riboprobes were detected using anti-DIG antibody (Roche). Embryos were stained with NBT/BCIP. *Shh* and *Msx2* riboprobes were gifted from A. McMahon, University of Southern California, Los Angeles, and R. Hill, The University of Edinburgh, Edinburgh, respectively.

BAC Modification and LacZ Staining. Modification of bacterial artificial chromosome (BAC) DNAs and LacZ staining were as previously described (2). The *LacZ* sequence with the hsp68 promoter was inserted at a HindIII site (Chr14: 110130495–110130500). Embryos were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde, and 0.2% Nonidet Nonidet P-40 at 4 °C for 1 h. After washing with PBS, the embryos were stained in a solution containing 0.5 mg/mL X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 0.2% Nonidet P-40 in PBS at 37 °C overnight.

CRISPR/Cas9 System. We adopted the CRISPR/Cas9 system previously reported for modification of the *Hm* genome (3). Oligonucleotides containing target sequences were annealed and inserted into pX330 plasmid that had been digested with BbsI and gel-purified (4) (#42230; Addgene). The T7 promoter was added to the Cas9 and sgRNA sequences in pX330 by PCR amplification. Purified PCR products were used as templates for in vitro transcription using a mMESSAGE mMACHINE ULTRA kit (Life Technologies) for Cas9 mRNA and a MEGAshortscript kit (Life Technologies) for sgRNAs. Transcribed RNAs were then purified using a MEGAClear kit (Life Technologies). Cas9 mRNA (100 ng/μL) and a pair of sgRNAs (50 ng/μL each) were mixed in injection buffer (5 mM Tris·HCl, pH 7.5, and 0.1 mM EDTA) and passed through a 0.22-μm filter. The mixture of Cas9 mRNA and sgRNAs was injected to the cytoplasm of fertilized eggs, which were then cultured in KSOM medium (Arc Resource) for 1 d and transferred into oviducts of MCH (ICR) recipient females. Forward primers and the common reverse primer are listed in Dataset S1.

qRT-PCR. Expression of *Shh* transcripts was assayed with SYBR Premix Ex TAq II (TaKaRa) and the Dicer real-time system (TaKaRa). Total RNA was isolated using an RNeasy mini kit (Qiagen). cDNA was synthesized using a Primescript RT reagent kit (TaKaRa). All PCR reactions were carried out in duplicate and validated by melting curve analysis. Primer sets used in this study are listed in Dataset S1.

Microarray. Interdigital regions 2 and 3 were dissected from E13.5 hind limb, and the samples from five littermates were pooled. Total RNA was then isolated using an RNeasy mini kit (Qiagen). For each genotype, three total RNAs were pooled in equal concentrations and analyzed by Gene Chip Mouse Genome 430 2.0 Array (Affymetrix). Expression level was normalized by Affimetrix Expression Console Software with the MAS5 algorithm.

Overexpression of *Chrd*. We generated *K14-Chrd* transgenic mice, following a previous study (47). Briefly, the *KRT14* promoter was cloned from genomic DNA of human Caco2 cells (5). The full-length coding sequence of *Chrd* was cloned from cDNA of E13.5 limb of C57BL/6JJcl. The *K14-Chrd* overexpression construct was injected into one-cell embryos of (C57BL/6JJcl × DBA/2J) F1.

Inverse PCR. Genomic DNA (500 ng) was digested by restriction enzymes at 37 °C overnight. After heat inactivation for 20 min at 65 °C, digested DNA was self-ligated at 37 °C for 2 h in a volume of 250 μL (T4 Ligase; TaKaRa). Fifty nanograms of ligated DNA was used as a template for PCR. Primer sets used in this study are listed in Dataset S1.

ATAC-Seq. Distal autopods of hind limb were isolated from 10 embryos at E13.5 and homogenized by Dounce tissue grinder with buffer containing 20 mM Tricine, pH 7.8, 25 mM Sucrose, 15 mM NaCl, 60 mM KCl, 2 mM MgCl2, and 0.5 mM Spermidine. Isolated nuclei (5×10^4) were resuspend with lysis buffer (10 mM Tris·HCl, pH 7.4, 3 mM MgCl2, and 0.1% IgePAL CA-630) and centrifuged at 900 *g* for 10 min at 4 °C. The nuclei pellet was resuspended in 50 μL TD buffer containing 2.5 μL Tn5 transposase (Illumina) and incubated at 37 °C for 30 min. Transposed DNA was purified with MinElute PCR Purification Kit (Qiagen). The ATAC-seq libraries were sequenced using the Illumina HiSeq 2500 in Rapid Run Mode with 131-cycle single-end sequencing. We mapped short reads to the reference genome of wild type using bowtie2 and called peaks using Model-based Analysis for ChIP-seq (MACS) and visualized with the UCSC Genome Browser (6). We picked up peaks with MACS scores above 400 in *Hm*.

1. Echelard Y, et al. (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75:1417–1430.
2. Tsukiji N, Amano T, Shiroishi T (2014) A novel regulatory element for *Shh* expression in the lung and gut of mouse embryos. *Mech Dev* 131:127–136.
3. Wang H, et al. (2013) One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. *Cell* 153:910–918.
4. Cong L, et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* 339:819–823.
5. Staggers WR, Paterson AJ, Kudlow JE (1995) Sequence of the functional human keratin K14 promoter. *Gene* 153:297–298.
6. Kent WJ, et al. (2002) The human genome browser at UCSC. *Genome Res* 12: 996–1006.

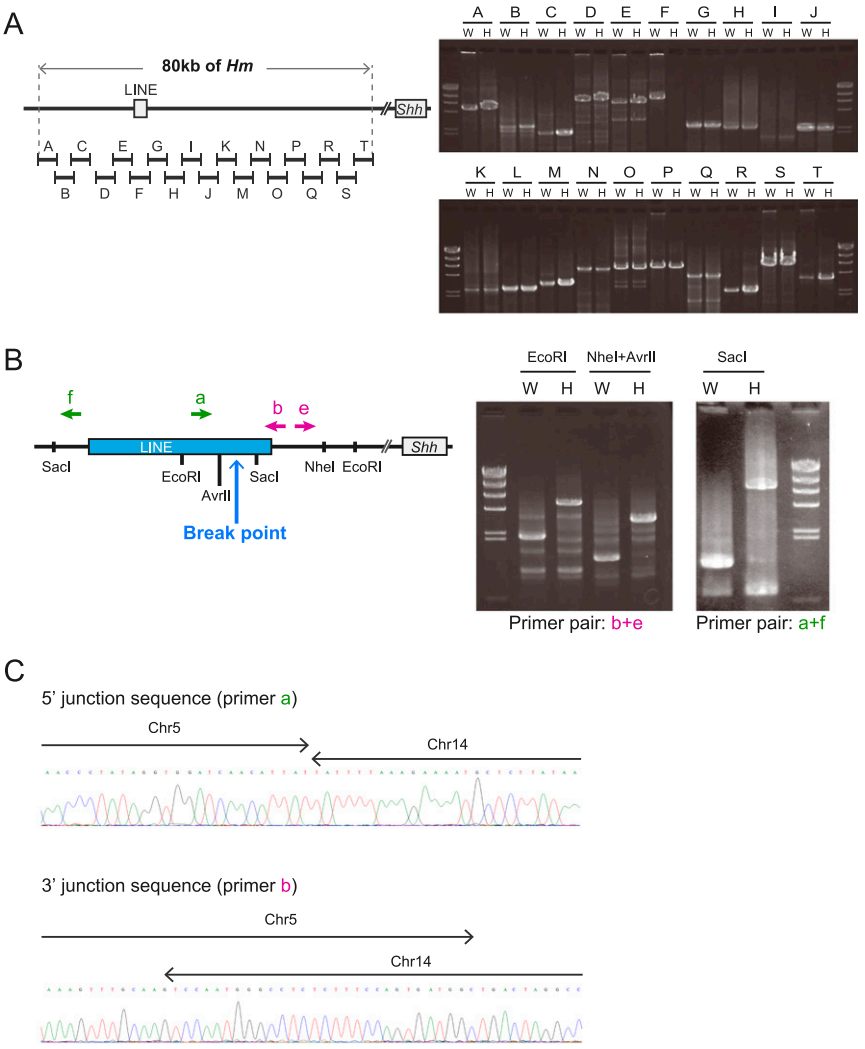

Fig. S1. Identifying a structural mutation of *Hm*. (*A*) Tiling genomic PCR amplicons. Amplified fragments are indicated below the *Hm* region. Note that only fragment F could not be amplified from the *Hm* homozygote, as shown in the agarose gel electrophoretic profiles on*Right*. H, *Hm* homozygote; W, wild type. (*B*) Inverse PCR for locating the mutation site. Restriction enzyme sites for DNA digestion and positions of primers for amplification after ligation are shown on *Left*. A break point was located between AvrII and SacI sites. The agarose gel electrophoretic profiles are shown on *Right*. H, *Hm* homozygote; W, wild type. (*C*) DNA sequence of the two junctions between Chr5 and Chr14 in *Hm*. At the 3′ junction, a 30-bp sequence was shared by both chromosomes. Primers a and b used in sequencing are identical to those shown in *B*. λ-HindIII was used as the DNA marker in *A* and *B*.

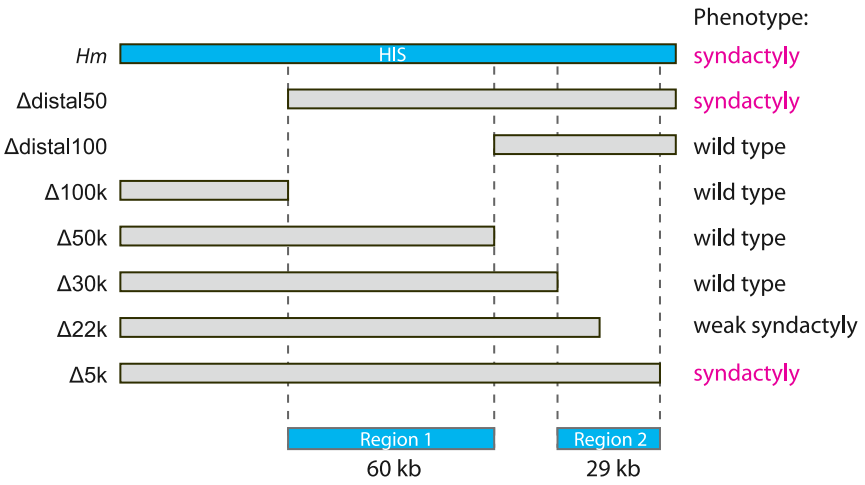

Fig. S2. A series of deletions of HIS in *Hm*. Top blue bar indicates the 150-kb HIS sequence. Gray bar indicates the remaining sequence after deletion. Phenotype of each deletion mutant and information to generate the HIS deletions are listed in Dataset S2.

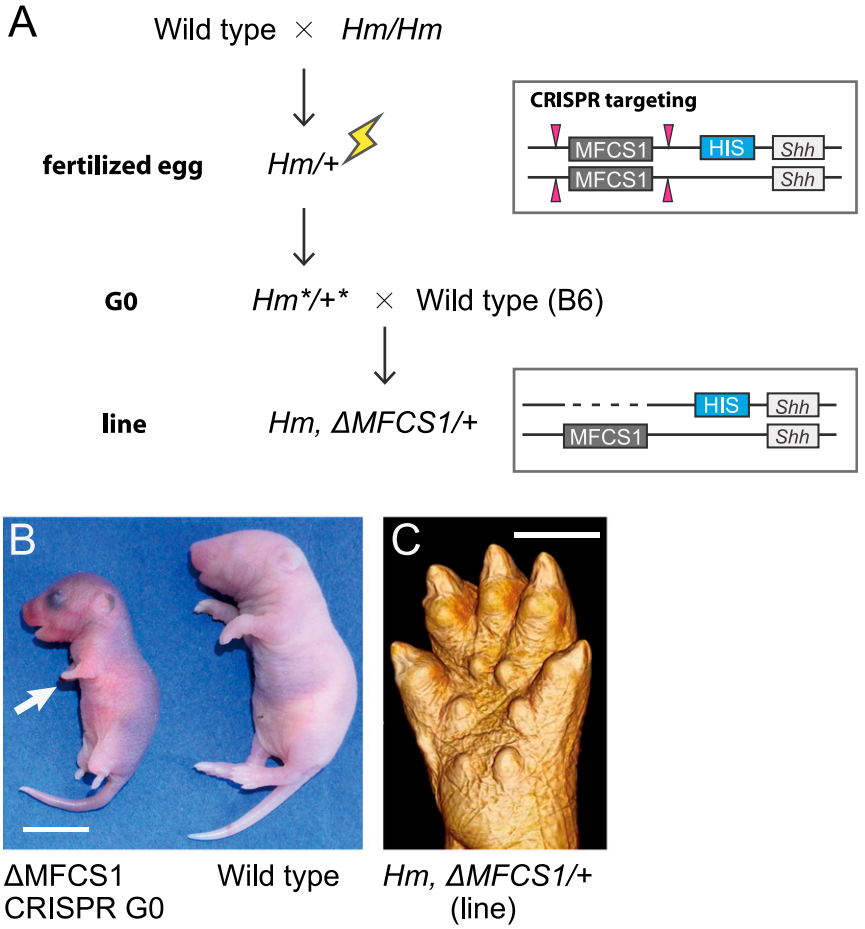

Fig. S3. Regulatory action of *Hm* is independent of the limb enhancer. (*A*) Schematic diagram of MFCS1 deletion using the CRISPR/Cas9 system. Magenta arrowheads indicate the target sites of Cas9. The mating scheme for elimination of MFCS1 is displayed. Wild-type eggs were fertilized in vitro with sperm from an *Hm/Hm* male, and the eggs were injected with *Cas9* mRNA and two guide RNAs. A G0 mouse was backcrossed to a wild-type mouse to establish the line *Hm, ΔMFCS1/+*. (*B*) Neonatal G0 pups of *Hm/+* that have a deletion of MFCS1 sequence, introduced by the CRISPR/Cas9 system. The white arrow indicates a truncated limb. The wild-type mouse was derived from an MCH (ICR) feeder mother. (*C*) Micro-CT image of right hind limb of *Hm, ΔMFCS1/+* at P0. (Scale bars, 1 cm in *B* and 1 mm in *C*.)

Other Supporting Information Files

Dataset S1 (XLSX)
Dataset S2 (XLSX)
Dataset S3 (XLSX)
Dataset S4 (XLSX)
Dataset S5 (XLSX)