Supp. Methods

Human DNA sequencing

Six family members were available for testing. Written informed consent was obtained from all participants or their legal guardians. The proband, her mother, father and sister were available for clinical examination. Genomic DNA was extracted from peripheral blood using standard methods (Qiagen kit EZ1). Primer sequences used for PCR and sequencing of the ZRS were obtained from Lettice et al. (Lettice, et al., 2003). Sequencing was performed using Big Dye terminator v3.1 cycle sequencing kit (Applied Biosystems) according to standard procedure and analyzed with the ABI-3730 DNA Analyzer (Applied Biosystems). The sequences were analyzed using Seqscape v2.5 (Applied Biosystems) and Sequencher (Gene Codes Corporation). The study was approved by the Regional Ethical Review Board in Stockholm, Sweden (protocol number 2010/193032) and the UCSF Committee on Human Research (protocol 10-03111).

Transcription Factor Binding Site Predictions

We acquired 345 transcription factor motifs from the UniPROBE database (Newburger and Bulyk, 2009) and compiled a small number of motifs for additional known limb factors from the literature including *TBX5* (MIM# 601602) (Ghosh, et al., 2001), *TBX6* (MIM# 602427) (White and Chapman, 2005), the *PBX-MEIS1* complex (MIM#s 602100, 601739) (Chang, et al., 1997) and the *MEIS1-HOXA9* (MIM# 142956) complex (Shen, et al., 1997). Binding sites were predicted by scanning DNA sequences for motifs using our implementation of the MATCH algorithm (Kel, et al., 2003) with a score threshold of 0.8.

Mouse Transgenic Analysis

The complete ZRS region was PCR amplified from patient II/1 with primers carrying *Xho*I and *Apa*I restriction sites (FwPrimer: GGCCctcgagTTTCAAATGCTCACTTTACATGG, RevPrimer: ATgggcccTGCTGAAGTGATACTGAAGAGAGG) and cloned into the *Hsp68-LacZ* enhancer assay vector (Kothary, et al., 1989). It was sequence-verified (Quintara Biosciences) to make sure that it had the ZRS603ins13 (NG-009240.1:g.106934_106935ins13) mutation and that no other mutations existed in the sequence. The wildtype enhancer assay vector was generated by removing the insertion from the ZRS603ins13 (NG-009240.1:g.106934_106935ins13) - *Hsp68-LacZ* vector (Mutagenix, Hillsborough, NJ, USA) and sequence verified. Generation of transgenic mice and Beta galactosidase staining at embryonic (E) day 11.5 were done by Cyagen Biosciences, Inc. (Guangzhou, China). All animal work was approved by the UCSF Institutional Animal Care and Use Committee (protocol AN084690).

Supp. References

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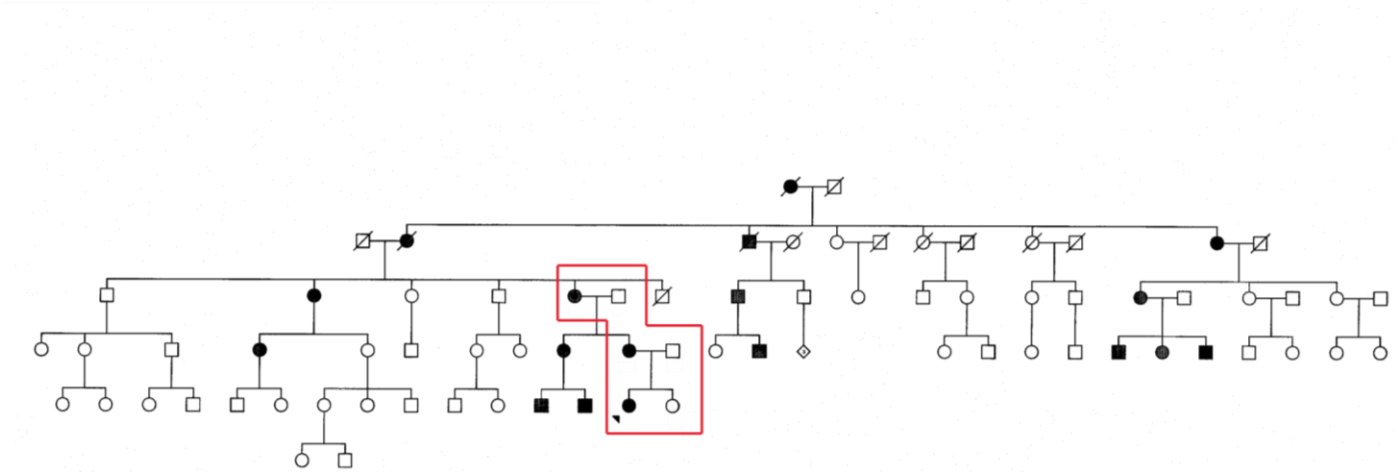
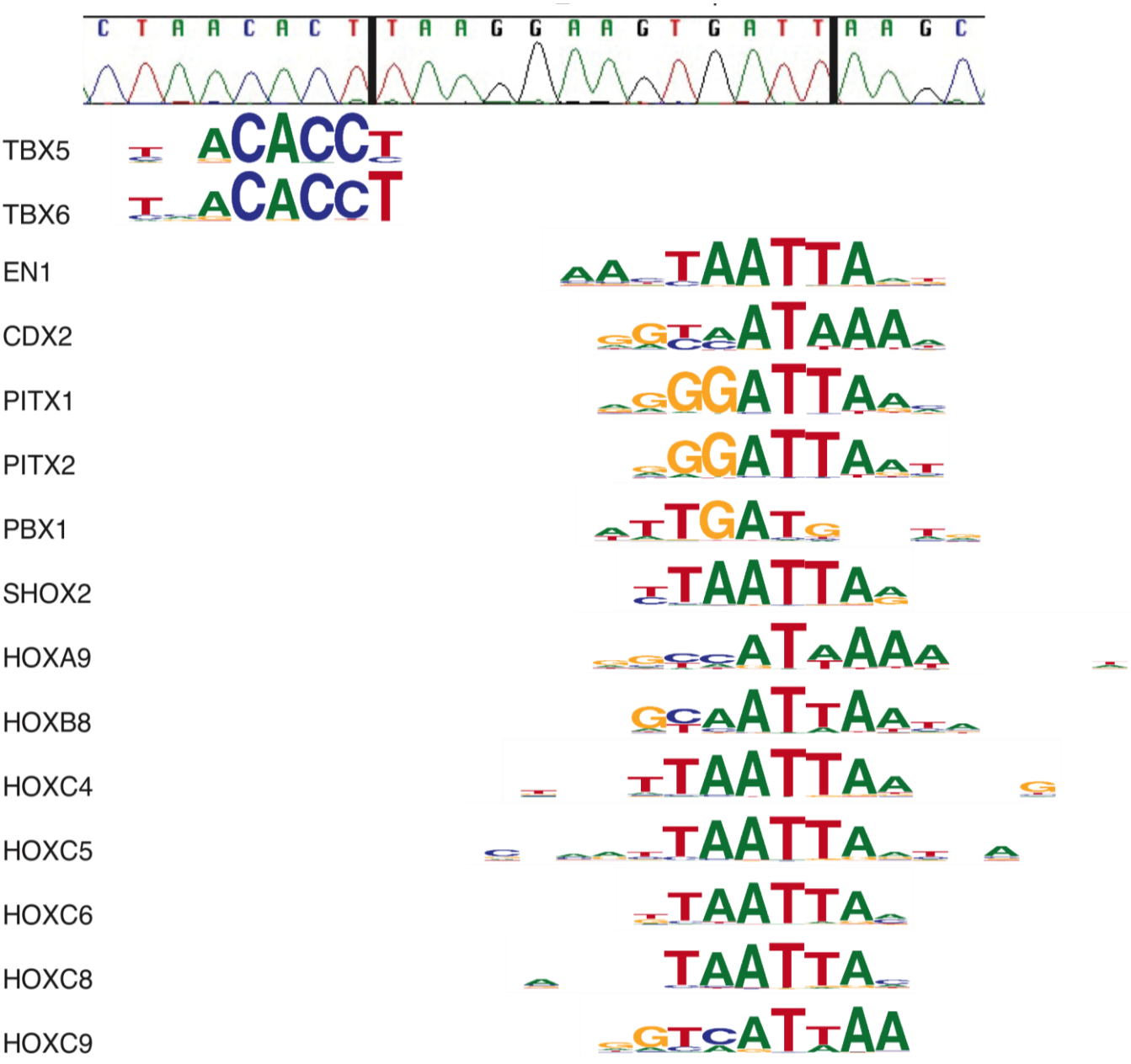
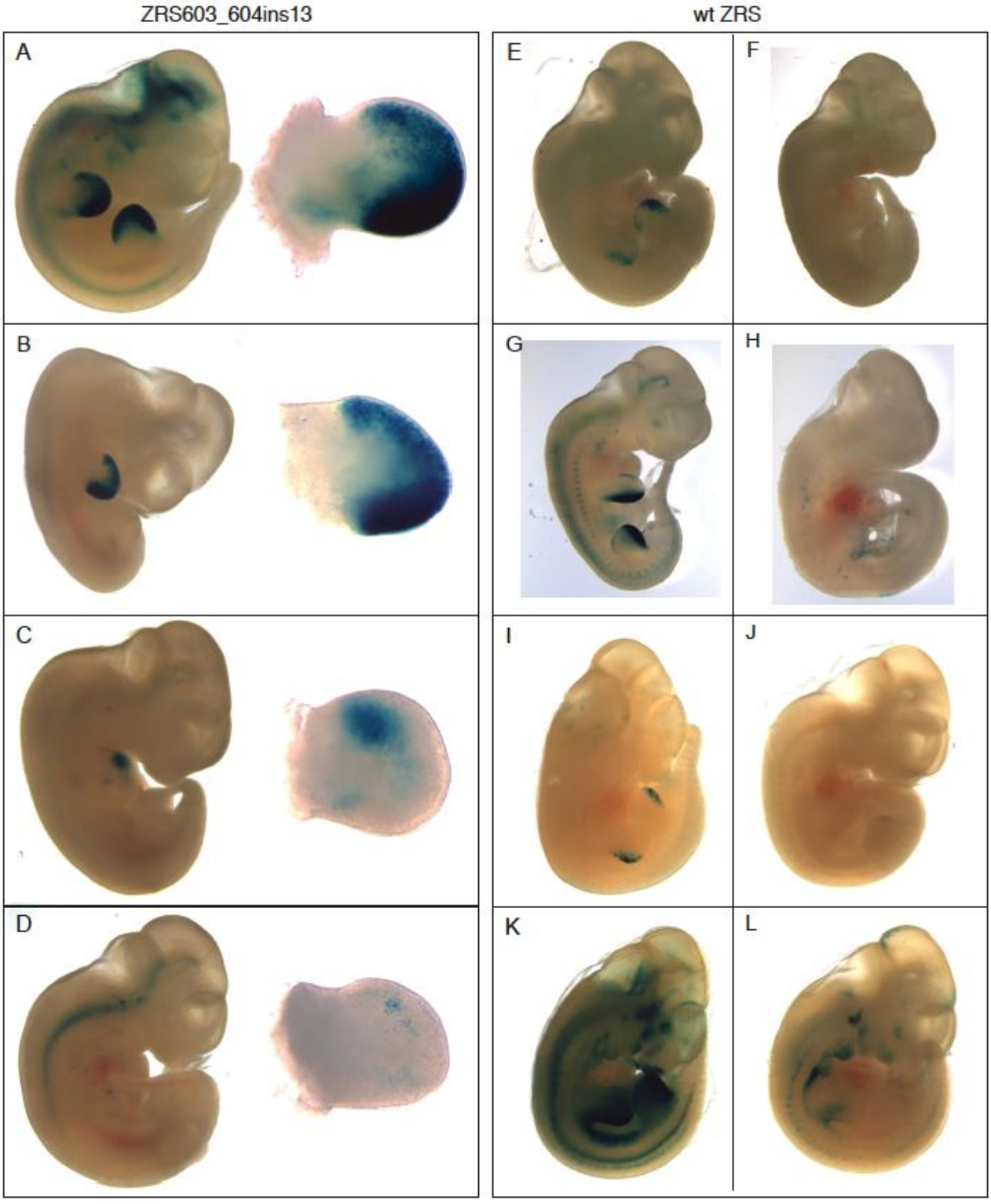
Supp. Figures



Supp. Figure S1. Full pedigree of the extended Swedish family with 18 affected individuals in 6 generations. The red box indicates the portion of the family shown in Figure 1A.



Supp. Figure S2. Transcription factor consensus binding motifs from UniProbe database and from literature sources are found in two clusters in the 13bp insertion (located between the dark bars on the chromatogram).



Supp. Figure S3. Whole-mount and dissected forelimbs show the anterior limb LacZ expression driven by ZRS603ins13 (NG-009240.1:g.106934_106935ins13) in multiple transgenic E11.5 mice compared to predominantly posterior expression driven by wtZRS. A. This embryo has strong expression in the anterior as well as posterior of both forelimbs and hindlimbs. B. This

embryo shows strong posterior and anterior limb expression in both forelimbs and weak expression in hindlimbs, which is difficult to see with the twisted orientation of the embryo. C. This embryo has strong staining only in the forelimbs with most expression in the anterior part of the limb. D. The fourth embryo has weak expression only in the forelimbs, but does show anterior limb expression. E-L. Seven of the eight (all except L) wtZRS transgenic embryos show some posterior limb expression of LacZ. E and G also show a small degree of anterior limb LacZ expression, but this is much weaker than the posterior expression, in contrast to all four of the mutant transgenic embryos (A-D).