Supplementary Materials and Methods

Constructing and Targeting the Conditional Hoxc8 Misexpression Allele

To make the conditional RosaCAGGSHoxC8IresGFP allele (herein abbr. CAGC8), the *Hoxc8* open reading frame was PCR amplified from E11.5 whole embryo mouse cDNA and cloned into pCAGstop2 along with an IresEGFP casette. The plasmid was then placed into the 3' UTR of the Rosa locus. LoxP sites between the CAG promoter and coding sequence allowed *Cre*-mediated removal of a transcription stop (and PGK-neomycin selection cassette) resulting in expression of bicistronic *Hoxc8* and *GFP* messages. Double heterozygote offspring created by crossing dams carrying the CAGC8 overexpression allele with males harboring any *Cre* driver allele will express *Hoxc8* and *GFP* throughout the lineage of *Cre* expressing cells.

Immunohistochemistry and X-gal

Briefly, paraffin sections were dewaxed and rehydrated into water for five minutes before antigen retrieval (15-30 minute boil in 0.1 M citrate phosphate, pH 6.0). Sections were blocked for endogenous peroxidase in 0.1% H_2O_2. Primary antibodies were diluted in CytoQ solution and incubated on sections overnight at 4°C. Vector Elite ABC mouse or rabbit kits were used to amplify signal. Vector DAB (brown) or SG (black) kits were used with brief hematoxylin (Fisher) or nuclear fast red (Sigma) counterstain to visualize nuclear localization. Primary antibodies on frozen sections were treated as per paraffin sections, but a secondary antibody linked to alexa-fluor fluorescent reporter (Invitrogen) was used at 1:1000 for fluorescent imaging and DAPI was used as a nuclear counterstain. Fluorescent and bright field images were obtained with a M205FA microscope, using AF6000 imaging software (both Leica Microsystems).

Whole mount embryos carrying a beta-galactosidase reporter (RosaLacZ or TOPgal) were processed for 90 minutes in formalin/glutaraldehyde fixative before staining in X-gal solution (4 hours at RT° or up to 16 hours at 4°C). Post-fixed embryos were photographed before embedding for vibratome (70µ) sectioning (in agar), or for paraffin (10µ) sectioning (in paraplast). X-gal stained E10.75 C8cre/LacZ frozen sections were processed post-sectioning.

Whole-Mount In Situ Hybridization

Tbx3 and *Wnt10b* in situ hybridization probes were amplified from E11.5 mouse cDNA using the following primers: Tbx3F: 5'- TACTGAAACCGACTTCCAGGAG -3'. R: 5'-ACATTCTCTTTGGCATTTCGG-3'; Wnt10bF: 5'- TCACAGAGTGGGTCAATGTG-3', R: 5'- GTGACTCTTTCAGGTGCTCC-3'. Products were cloned into TOPOII dual promoter kit (Invitrogen K4500-01), linearized, then transcribed to make labeled antisense RNA probes using the appropriate polymerase with DIG RNA Labeling Kit (Roche 11 175 025 910). *Hoxc8*, *Myf5*, *Fgf10*, and *Bmp2* antisense probes were similarly prepared from frozen plasmid stock. All plasmids are available on request.

Chromatin Immunoprecipitation (ChIP)

In silico anaylsis (Kent et al., 2002) (http://genome.ucsc.edu/ ; Dec. 2011 GRCm38/mm10) was performed to identify putative *Hox* transcription factor binding sites contained within or near regions of evolutionary sequence conservation flanking and

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intronic to the *Tbx3* coding sequence (Fig. 9B). Ten specific primer sets were designed to anneal at 60°C and amplify ten short (~150 bp) conserved or control regions (Table S1). The ChIP procedure we employed was a modification of a video protocol developed for E8.5 embryos, available at the Jove website (Cho et al., 2011). Briefly, E11.5 W6cre/CAGC8 and control embryos were harvested from CAGC8 females crossed to W6cre males. Lateral flank (mesoderm + ectoderm) and dorsal tissue (consisting of somites + neural tube) were separately collected. Left and right sides from three embryos were used to prepare flank and dorsal ChIP samples of each genotype (W6cre/CAGC8 and control). Tissue was disaggregated in Collagenase Type II (Gibco #17101-015). Approximately 5 X 10^6 cells were used for each chromatin preparation. Samples were cross-linked, washed in DPBS, and frozen at -80°C until further use.

Thawed cross-linked cells were suspended in 200 µL SDS lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA and 1% SDS) with freshly added PIC (Roche #11 836 170 001). These were sonicated (30 seconds on, 30 seconds off) for 30 cycles using an automated Diagenode bioruptor sonication system (B01010002). After centrifugation, 40 µL of each eluate was aliquoted into 5 equal volumes and frozen at -80°C until further use. Thawed sonicated samples were diluted in 400 µL ChIP dilution buffer (16.7 mM Tris-HCl (pH 8.1), 1.2 mM EDTA, 1.1% TritonX, 167 mM NaCl and 0.01% SDS). Immunoprecipitation (IP) and washing steps were performed with a Dynal MPC magnetic particle concentrator (Invitrogen 123-210), using magnetic Protein G dynabeads (Novex #10007D) which had been blocked with 5 mg/mL BSA fraction V, 20 mg/mL glycogen, and 20 mg/mL yeast tRNA. Half of each sample was IP'd with blocked beads and Hoxc8 antibody (Covance) and the other half with blocked beads and mouse IgG serum (Vector Laboratories). Chromatin-protein-bead complexes were washed twice in dialysis buffer at 4°C (50 mM Tris-Cl (pH 8.1), 2 mM EDTA), four times in ChIP wash buffer at 4°C (100 mM Tris-Cl (pH 8.0), 500 mM LiCl, 1% NP-40, 1% NaDOC), and once in TE at RT°. Chromatin was eluted with 2 x 150 µL elution buffer (50 mM $NaHCO_3$, 1% SDS) at 65°C. Chromatin cross-links were reversed (in ChIP samples as well as pre-IP input), by incubating samples for four hours at 65°C with 20 µL 5 M NaCl and 1 µL 10 mg/mL RNAseA. Chromatin was purified with Qiagen PCR columns and eluted in 50 µL Qiagen EB buffer. 40 cycle PCR reactions were seeded with 2 µL flank or dorsal chromatin. (Table S1). Only primer pair #5 amplified both flank and dorsal tissue that had been precipitated with Hoxc8 antibody, and failed to amplify mouse IgG precipitated chromatin. This primer pair was then successfully tested on chromatin obtained from W6cre/CAGC8 embryos.

Reference

Kent, W. J., Sugnet, C. W., Furey, T. S., Roskin, K. M., Pringle, T. H., Zahler, A. M. and Haussler, D. (2002). The human genome browser at UCSC. *Genome Res* **12**, 996-1006.

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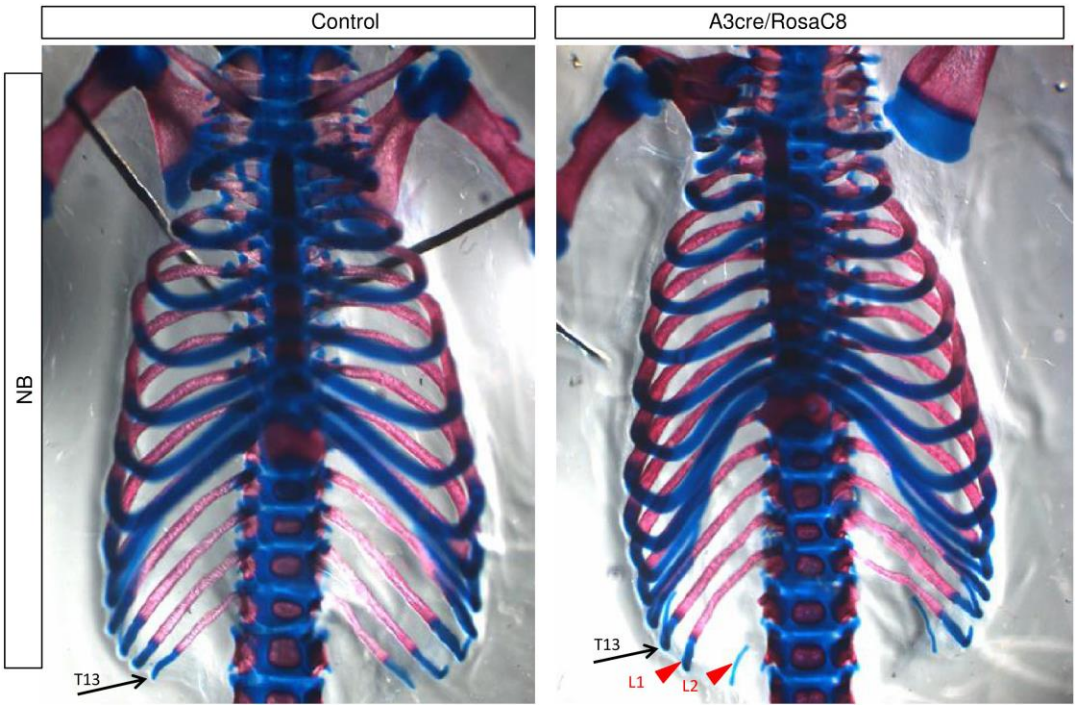

Figure S1
Ribs in newborn control and mutant mice generated from our initial *Hoxc8* conditional misexpression construct (crossed to Hoxa3IresCre). This initial construct lacked the CAGGS promoter and was instead driven by the endogenous Rosa promoter. Mutants survive until birth and have a considerably milder rib phenotype than A3cre/CAGC8 embryos. Arrows point to T13, arrowheads point to ectopic ribs on the first two lumbar vertebrae (T14 and T15).

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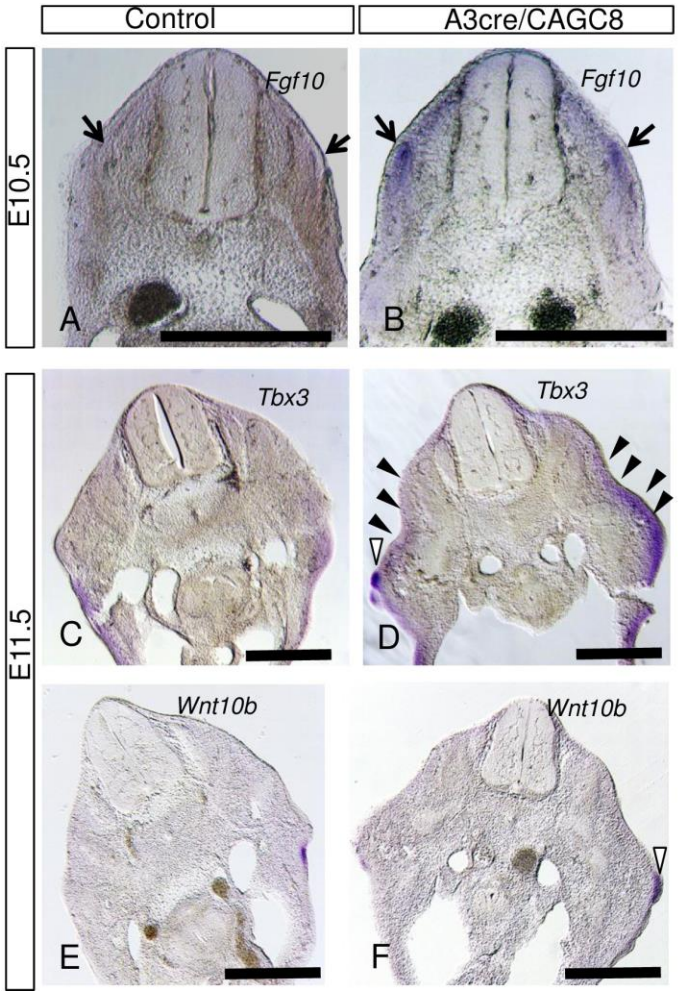

Figure S2
70 µm vibratome sections through the cervical region of whole mount control (A,C,E) and A3cre/CAGC8 (B,D,F) in situ embryos. **(A,B)** E10.5 embryos probed with *Fgf10*. Arrows indicate somitic upregulation in mutant. **(C,D)** E11.5 embryos probed with *Tbx3* black arrowheads indicate upregulated mesodermal signal in mutant. White arrowhead indicates an ectopic cervical placode. **(C,D)** E11.5 embryos probed with *Wnt10b*. Arrowhead indicates an ectopic cervical placode. Scale bars: 500µm.

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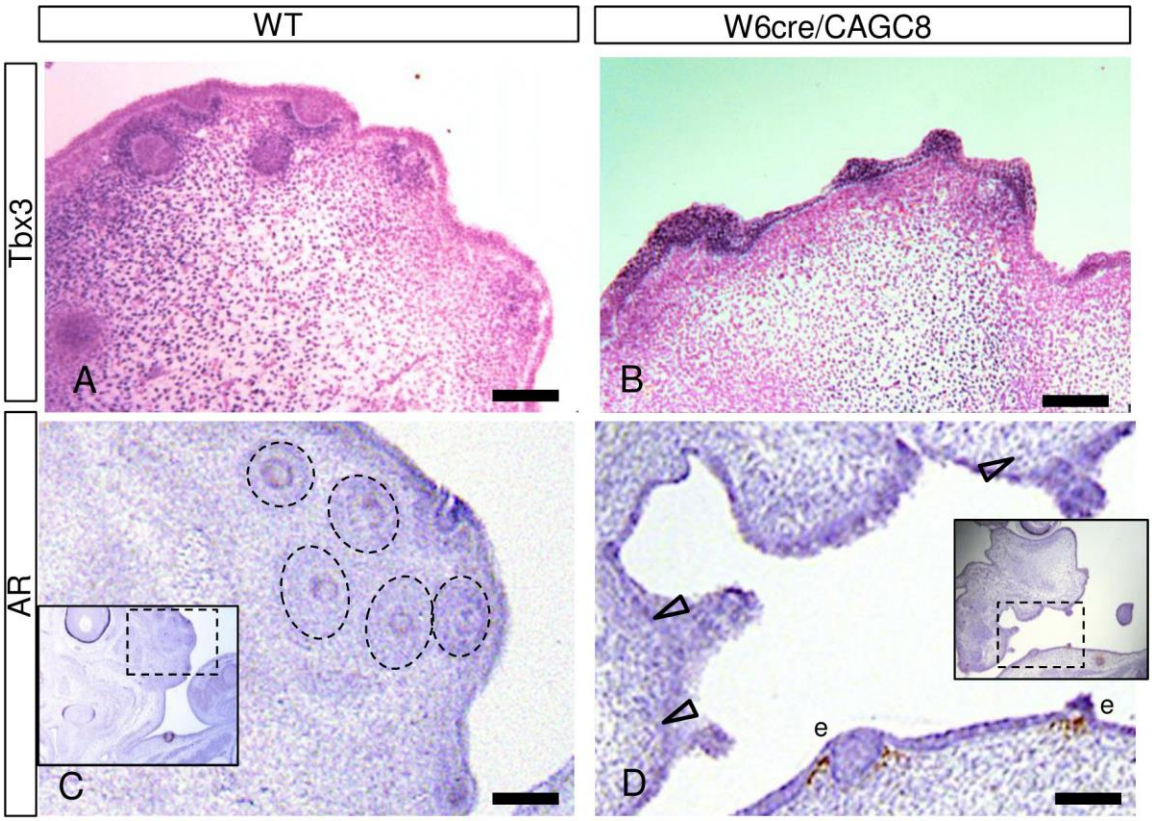

Figure S3
(A,B) Tbx3 is strongly upregulated in the ectoderm of defective W6cre/CAGC8 vibrissal placodes. **(C,D)** Lack of androgen receptor (AR) in mesenchyme of mutant vibrissae (open arrowheads) suggests defective vibissae are not reprogrammed towards a mammary fate. Scale bars: 100μm.

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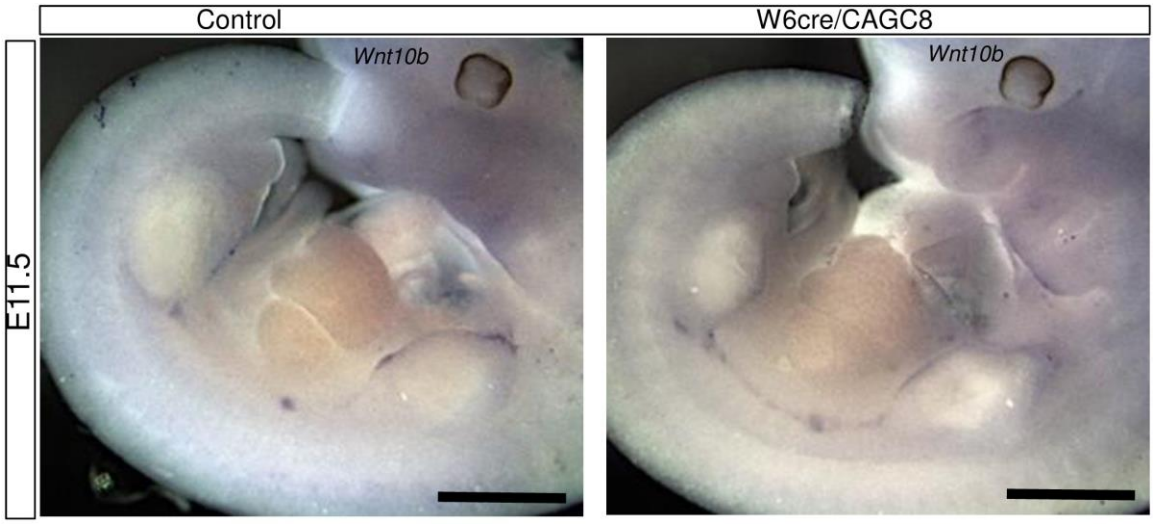

Figure S4
Delayed mammary placode assembly and lingering *Wnt10b* expression along the mammary line of an (early) E11.5 W6cre/CAGC8 embryo compared to a control littermate. Limbs are removed. Scale bars: 1mm

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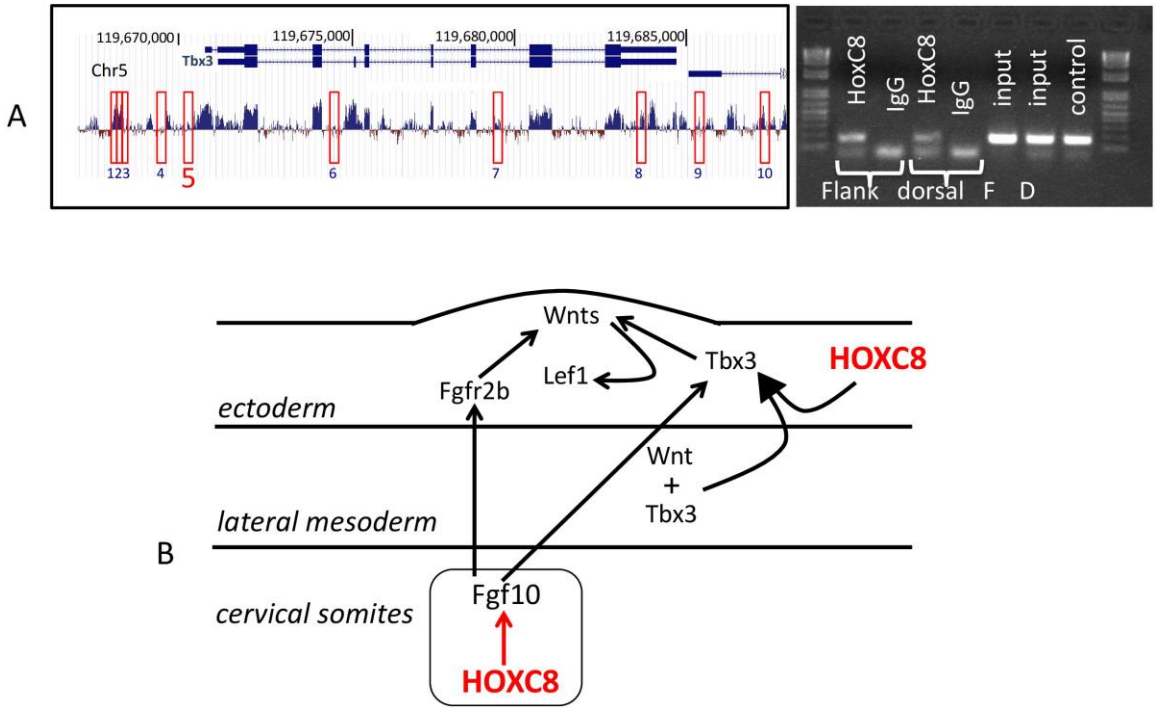

Figure S5

(A) Eight evolutionarily conserved genomic regions (excluding exons) designated by boxes 1, 2, 3, 5, 7, 8, 9, 10 and two non-conserved regions (boxes 4, 6) associated with the *Tbx3* locus were identified as carrying putative *Hox* transcription factor binding sites (see supplementary materials and methods). Polymerase chain reaction (PCR) amplified a region 1.5 Kb 5' of the *Tbx3* ATG start (region/primer set #5) from Hoxc8 antibody-immunoprecipitated tissue but not from mouse IgG serum-immunoprecipitated tissue of a W6cre/CAGC8 mutant. **(B)** Model of ectopic mammary placode formation requires somitic HoxC8 expression for induction of *Fgf10* and ectodermal Hoxc8 expression for induction of *Tbx3* (see results and discussion).

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Table S1
Tbx3 primer sequences for ChIP. Only primer set #5 amplifies sequence bound to Hoxc8 antibody, but not to mouse IgG serum.

Primer set	Forward	reverse
1	5'-gccacaagcctaagcaagac-3'	gatcaaaagcaggaaggtgc
2	gcaccttcctgcttttgatcc	atcccagtttgccacttctc
3	agaagtggcaaactgggatg	gcatgcaaataatctggcct
4	caaaggtcttgtcccaggaa	cgatcagactttggttgggt
5	**cgcaggagctagaggatctg**	**tctgcagcttcttccctttc**
6	gaatgtggacagacagggct	tctgacttttcacccaggc
7	gtgtcagctttggggaggta	tcttccaccacaccctcttc
8	gggagatgaagtcctgtgga	ccagcatcggctcttaaaac
9	agtcccgagtcagttaggca	aggacaggacagaggcttca
10	gggctttagagctgtgggta	agcctacacaccgtacaccc

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