HOXC8 initiates an ectopic mammary program by regulating Fgf10 and Tbx3 expression, and Wnt/β-catenin signaling

Lara S. Carroll¹ and Mario R. Capecchi²

¹ Moran Eye Center, University of Utah, Salt Lake City, UT 84132
² Department of Human Genetics and Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT, 84112

*Author for correspondence: lsc3@utah.edu

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SUMMARY
The role of Hox genes in the formation of cutaneous accessory organs such as hair follicles and mammary glands has proved elusive, likely owing to overlapping function and expression among various homeobox factors. Lineage and immunohistochemical analysis of Hoxc8 in mice revealed that this midthoracic Hox gene has transient, but strong regional expression in ventro-lateral surface ectoderm at E10.5, much earlier than previously reported. Targeted mice were generated to conditionally misexpress Hoxc8 from the Rosa locus using select Cre drivers, which significantly expanded the domain of thoracic identity in mutant embryos. Accompanying this expansion was the induction of paired zones of ectopic mammary development in the cervical region, which generated between three and five pairs of mammary placodes anterior to the first wild type mammary rudiment. These rudiments expressed the mammary placode markers Wnt10b, and Tbx3 and were labeled by antibodies to mammary mesenchyme markers ERα and AR. Somitic Fgf10 expression, which is required for normal mammary line formation, was upregulated in mutant cervical somites, and conditional ablation of ectodermal Tbx3 expression eliminated all normally positioned and ectopic mammary placodes. We present evidence that Hoxc8 participates in regulating the initiation stages of mammary placode morphogenesis, and suggest that this and other Hox genes are likely to have important roles during regional specification and initiation of these and other cutaneous accessory organs.

INTRODUCTION
During embryonic development, the epidermis and underlying dermis of vertebrate skin collaborate via respective epithelial and mesenchymal signals to create cutaneous appendages, such as hair and feather follicles, mammary glands, teeth, and sweat glands. Despite the morphological and functional differences among mature skin organs, each begins as a placode, a raised epithelial thickening that initiates in response to a broadly expressed Wnt signal from the dermis (Mikkola, 2007). As mammary and hair placodes begin to develop and invade the mesenchyme, dermal and epidermal Wnt signaling continues, along with additional signaling molecules such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), ectodysplasin (Eda-A1) and Sonic Hedgehog (SHH) to effect specific development of each organ type. (Andl et al., 2002; Chu et al., 2004; Gallego et al., 2002; Mikkola and Millar, 2006; Mustonen et al., 2004; Petiot et al., 2003; Plikus et al., 2004; St-Jacques et al., 1998; Veltmaat, 2013; Zhang et al., 2009).

In mice, the first visible sign of mammary development is the appearance at E10.5 of two histologically distinct lines of pseudostratified ectoderm between the fore- and hindlimb buds, marked by Wnt10b expression (Veltmaat et al., 2004). This ectoderm is a permissive region for mammary rudiments (MRs) 2, 3, and 4, joining additional streaks of mammary permissive ectoderm in the axial and inguinal regions giving rise to MRs 1 and 5 (Veltmaat et al., 2004). Ectopic mammary glands occur most commonly at inappropriate sites along these lines. Evidence in rabbits and mice suggests that mammary placodes form by migration of epithelial cells into and along the mammary lines resulting in the five pairs of mammary rudiments developing non-sequentially at characteristic positions along the body axis (Lee et al., 2011; Propper, 1978). Molecular requirements differ among the pairs of mammary placodes, and
differential gene expression profiles may underlie some of the heterogeneous attributes and susceptibilities to tumor incidence in adult mammary glands (Veltmaat et al., 2013).

Proper positioning of the mammary line along the dorso-ventral axis is achieved in part by mutual antagonism between ventrally expressed Bmp4 and more dorsally expressed T-box transcription factor, Tbx3 (Cho et al., 2006; Veltmaat et al., 2006). These and additional mammary factors such as Gli3, retinoic acid, Nrg3 and Fgf10 play important roles in the appropriate patterning of Wnt signals which are required to achieve proper rostro-caudal positioning of placodes (Cho et al., 2012; Cowin and Wysolmerski, 2010; Hatsell and Cowin, 2006; Howard, 2008; Lee et al., 2013; Mailleux et al., 2002; Veltmaat et al., 2006).

The idea that a “HOX code” (Kessel and Gruss, 1991) might underlie regional distribution of cutaneous appendages has been around since the discovery that Hox gene expression exhibits positional variation within the skin itself (Bieberich et al., 1991; Chuong, 1993). A majority of Hox genes appear to be expressed in fetal or adult skin and hair follicles (Awgulewitsch, 2003; Johansson and Headon, 2014), and several Hox genes are expressed in developing and mature mammary glands, or become dysregulated during mammary neoplasia (Chen and Sukumar, 2003; Hayashida et al., 2010; Lewis, 2000; Wu et al., 2006). During early embryogenesis, expression of vertebrate Hox genes initiates in a rostral to caudal direction along the body axis in a sequence mirroring the linear position of each gene within the four chromosomal Hox clusters, a unique feature termed “spatio-temporal colinearity”. Each cell along the Hox trajectory receives a distinct combination of HOX proteins, a “HOX code” (Kessel and Gruss, 1991) which may uniquely specify its position, patterning individual elements from the hindbrain to the most posterior vertebrae. However, unlike axial Hox expression, only a subset of tested Hox genes have been shown to have regional restriction of expression within mouse, human, or chick embryonic skin, including Hoxc8, Hoxb4, Hoxa7, Hoxd9, Hoxd11, and Hoxd13 (Kanzler et al., 1994; Reid and Gaunt, 2002). Several others, including Hoxc13, which has a critical role in hair shaft development, are expressed broadly throughout the epidermis and/or dermis (Godwin and Capecchi, 1998; Kanzler et al., 1994; Reid and Gaunt, 2002). Therefore, Hox temporal and spatial expression in embryonic skin does not strictly match the colinear expression found in axial Hox domains, and the putative HOX code responsible for globally defining domains of emerging epidermal organs has proved elusive, likely owing to the complex combinatorial nature of Hox expression and function.

The strongest evidence for Hox mediated regional patterning of epidermal organs comes from two thoracic Hox genes. Adult thoracic mammary glands of mice lacking functional Hoxc6 are devoid of mammary epithelium whereas inguinal mammary glands develop ductal structures and are less severely affected (Garcia-Gasca and Spyropoulos, 2000). Hoxc8 has been indirectly implicated in the specification of feather and hair types (Kanzler et al., 1997; Mentzer et al., 2008), and in mice, Hoxc8 shows regionally restricted expression during the first wave of hair placodogenesis, the earliest reported expression of any Hox gene in epidermis (Johansson and Headon, 2014; Kanzler et al., 1994).

Using a Hoxc8iresCre mouse line (Wu et al., 2008), we found Hoxc8 lineage in mammary line ectoderm by E10.75 and incorporated into all five mammary rudiments by E12.5. This result prompted us to carefully reexamine Hoxc8 expression in embryonic skin in order to assess the potential of this Hox gene to mediate early skin regionalization and skin appendage specification. Further analysis
demonstrated transient regionally specific expression of Hoxc8 protein in the ectoderm during mammary line formation, prior to the earliest reported Hoxc8 ectodermal expression. We tested the possibility that Hoxc8 expression plays a role in mammary line specification using mice carrying a targeted allele designed to conditionally express Hoxc8. Conditional misexpression of Hoxc8 using two out of three Cre drivers consistently led to the appearance of supernumerary mammary rudiments within two distinct domains: along the normal mammary line of mutant mice, and within the cervical region, anterior to the first mammary rudiment. These ectopic rudiments express placode markers Bmp2, Wnt10b and Tbx3 and are labeled by the mammary mesenchyme specific markers estrogen receptor alpha (ERα) and androgen receptor (AR). This study is the first to implicate a Hox gene in rostro-caudal positioning of mammary line ectoderm and placodes. We present evidence that Hoxc8 positively regulates Tbx3 and Fgf10 expression and Wnt/β-catenin signaling, and that moreover, Tbx3 is a direct Hoxc8 transcriptional target. These data further support the existence of a HOX code underlying regional specification of embryonic skin at the earliest stages of skin placode initiation.

MATERIALS AND METHODS
Detailed descriptions of the conditional RosaCAGGSHoxC8IresGFP mouse, immunohistochemistry, X-gal, in situ hybridization and Chromatin Immunoprecipitation (ChIP) protocols are provided in supplemental methods.

Animals and Genotyping
CAGC8 founders, as well as Cre driver lines: HoxC8IresCre (C8cre) (Wu et al., 2008), HoxA3Irescre (A3cre) (Macatee et al., 2003), Wnt6Irescre (W6cre) (Makki, 2010), Pax3cre (Engleka et al., 2005), reporter lines: RosaLacZ, RosaYFP (Soriano, 1999), and TOPgal (DasGupta and Fuchs, 1999) and a Tbx3floxp conditional knockout line (Frank et al., 2013) were maintained on C57BL6 or C57BL6 x CD1 genomic backgrounds. Mice and embryos were genotyped by PCR (primers available on request). To create experimental and control embryos for analysis, A3cre, Pax3cre, or Wnt6cre males were bred to CAGC8 females to create control and double heterozygote mutant littermates. Mutant and controls were easily distinguished by GFP signal using a fluorescent lamp. To generate control and W6cre/CAGC8 embryos with one or two conditionally ablated copies of Tbx3 in the Wnt6 domain, CAGC8/+; Tbx3floxcr or CAGC8/+; Tbx3flox dams were bred to W6cre/++; Tbx3floxcr or Tbx3flox dams were bred to W6cre/+; Tbx3floxcr mice. These males were morphologically and reproductively indistinguishable from littermates carrying no Cre allele. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Utah.
**Immunohistochemistry**

Embryos were fixed at 4°C in 4% PFA/PBS for 1-5 days (for paraffin embedding), or up to 24 hours (for OCT embedding). Primary antibodies used for this study were: Hoxc8 (1:200 Covance), Tbx3 (1:200, a generous gift from A. Moon, University of Utah), Lef1 (1:1,000; Cell signaling), AR (1:200; Millipore) and ERα (1:200; Santa Cruz Biotechnology).

**Whole-Mount In Situ Hybridization**

In situ hybridization was performed according to previous protocols (Boulet and Capecchi, 1996). Tbx3, Wnt10b, Hoxc8, Myf5, Fgf10, and Bmp2 probes are available on request.

**Chromatin Immunoprecipitation (ChIP)**

The ChIP procedure we employed was a modification of a Jove video protocol developed for E8.5 embryos (Cho et al., 2011).

**RESULTS**

**Hoxc8 is transiently expressed in ventrolateral flank ectoderm prior to formation of the mammary line**

Hoxc8 is cited as one of the first Hox genes expressed in embryonic mouse skin, with its earliest reported expression in E14.5 epidermis (Awgulewitsch, 2003; Johansson and Headon, 2014; Kanzler et al., 1994). Lineage analysis and Hoxc8 antibody were both employed to re-examine cutaneous Hoxc8 expression to determine if it is appropriately staged to play a role in early specification of mammary glands. Lineage was examined using a HoxC8IresCre mouse line (Wu et al. 2008) and either RosaYFP or RosaLacZ (C8cre/YFP and C8cre/LacZ) (Soriano, 1999). C8cre/YFP lineage at E12.5 is broadly represented throughout flank caudal to the forelimb bud (n=3). Reporter additionally labels all mammary rudiments (Fig.1A; MR1 is obscured by the forelimb) revealing that Hoxc8 protein is also expressed in early surface ectoderm and may be present in ectodermal precursors giving rise to mammary epithelium. Hoxc8 antibody did not label E13.5 mammary bud ectoderm (Fig.1B and data not shown, n=3), indicating that ectodermal Hoxc8 is transitory, preceding the mammary bud stage.

To pinpoint the timing and extent of transient Hoxc8 expression, we next examined Hoxc8 lineage in sectioned C8cre/LacZ embryos (in which all Hoxc8 expressing cells and descendants are labeled), and subsequently examined transient expression in wild type embryo sections labeled with Hoxc8 antibody (Fig. 1C-F and data not shown; 2-3 embryos were examined at each time point indicated.) LacZ reporter extends rostrally in E10.75 ventro-lateral ectoderm to the forelimb level encompassing the region of the developing mammary line between fore and hindlimbs (Fig. 1C). At a slightly earlier stage, Hoxc8 antibody labels all surface ectoderm extending between and including the forelimb and hindlimb buds of E10.5 embryos, which necessarily includes the rostro-caudal extent of the forming mammary line (Fig.1D and data not shown). By E11.5 and E12.5, ectodermal expression of Hoxc8 is considerably reduced, particularly in the epithelium of the forming mammary placodes and buds (Fig. 1E,F). In situ hybridization of wild type
embryos with a Hoxc8 probe (Fig. 1G; n=2) shows expression in E11.5 hypaxial extensions of thoracic somites which include S15 and S16, underlying the portion of the mammary line specifically giving rise to MR3 (Veltmaat et al., 2006).

**Rostral expansion of mammary ectoderm in A3cre/CAGC8 embryos accompanies expansion of thoracic vertebral identity.**

The paraxial and surface ectodermal expression of Hoxc8 make it an ideal Hox candidate for a potential role in mediating mammary line and 3rd placode specification. We shifted the domains of Hoxc8 paraxial, mesodermal, and ectodermal expression using the HoxA3IresCre mouse (Macatee et al., 2003) to test if Hoxc8 misexpression could alter mammary line and placode positioning. E10.5 A3cre/LacZ lineage shows widespread expression throughout lateral mesoderm and somites (the rostral expression limit of somitic Hoxa3 corresponds to the first cervical vertebrae) and much of the body ectoderm caudal to the 2nd branchial arch (Fig. 2A; n=3). A3cre/CAGC8 embryos express ectopic Hoxc8 wherever iresGFP signal is present in a pattern equivalent to A3cre/LacZ lineage. This fluorescent signal was subsequently used to genotype A3cre/CAGC8 mutants (Fig. 2B).

In contrast to the wild type pattern of 13 thoracic ribs seen in control embryos, mutant A3cre/CAGC8 embryos exhibited well-formed ectopic ribs on all cervical, lumbar and sacral vertebrae as well as rib-like extensions on several caudal vertebrae (Fig. 2C,D; n=6) This phenotype is 100% penetrant in A3cre/CAGC8 mutants and is consistent with previous studies demonstrating a fundamental role of Hox genes in assigning antero-posterior vertebral identity (Carapuco et al., 2005; Le Mouellic et al., 1992; McIntyre et al., 2007; van den Akker et al., 2001; Wellik and Capecchi, 2003). The cervical region appears elongated in A3cre/CAGC8 mutants, likely related to its transformation to a thoracic identity. Interestingly, we found that the rib phenotype is dependent on Hoxc8 dosage, as a nearly identical construct minus the potent CAGGS promoter (also targeted to the Rosa locus, driven only by the Rosa promoter) yielded only mild skeletal phenotypes, consistent with a previously reported Hoxc8 transgenic mutant (Pollock et al., 1992) (Supplemental Fig. 1; n=16).

The dermomyotome, marked by Myf5 expression, exhibited ectopic hypaxial extensions within the cervical and lumbar regions of E11.5 A3cre/CAGC8 embryos (Fig. 2E,F; n=3). We used Bmp2 as a general placode marker, as its expression focally marks epithelium of the mammary bud (Phippard et al., 1996), hair (Suzuki et al., 2009) vibrissae, tooth (Bitgood and McMahon, 1995), and tongue papillae (Jung et al., 1999). At E11.5, we found that A3cre/CAGC8 embryos exhibited strong upregulation of Bmp2. Focal Bmp2 expression was seen within irregularly spaced placodes in the cervical ectoderm, with dark streaks of Bmp2 signal along the normal mammary line (Fig. 2G,H; n=3). Control embryos exhibited only faint streaks of ectodermal Bmp2 expression along the mammary line, punctuated by focal upregulation within MR3, the first mammary rudiment to form. We next examined expression of three of the earliest known genes associated with mammary line/placode formation, Fgf10, Tbx3, and Wnt10b (Fig. 2I-R; n ≥3 per genotype per timepoint for each probe). Formation of placodes 1, 2, 3 and 5 requires Fgf10 expression, which emanates from thoracic somites. Homozygous ablation of Fgf10 or its ectodermal receptor, Fgfr2b results in the absence of all four placodes (Mailleux et al., 2002; Veltmaat et al., 2006). Fgf10 signal appears upregulated in cervical and thoracic somites of A3cre/CAGC8 mutant embryos at E10.5 compared to control littermates (Fig. 2I,J, and Supplemental Fig. 2A,B). Other domains of Fgf10 expression, including limb buds, appear unchanged in mutants.
Tbx3 is required for the formation of mammary buds 1, 3, 4, and 5 (Davenport et al., 2003; Eblaghie et al., 2004; Veltmaat, 2013). In humans, heterozygous mutation of the Tbx3 gene causes Ulnar-Mammary syndrome, characterized by upper limb, genital, mammary and other glandular defects (Bamshad et al., 1999). In both control and A3cre/CAGC8 E10.5 embryos, Tbx3 is expressed in a broad strip of lateral plate mesoderm underlying the mammary line, and in another broad region of lateral mesoderm extending caudally from the 4th pharyngeal arch to the forelimb (Fig. 2K,L). By E11.5, Tbx3 levels in control embryos have greatly decreased in the cervical lateral mesoderm, but remain high in the A3cre/CAGC8 mutant, as a continuum extending from the hindlimb through thoracic and cervical levels (Fig. 2M,N and Supplemental Fig. 2C,D). Focal Tbx3 upregulation in mutant cervical placodes is obvious by this stage. However, in the mammary line ectoderm, mutant Tbx3 signal persists as a streak, while placode formation in controls is nearly complete (Fig. 2M,N). Notably, ectopic cervical placodes are not restricted to a linear pattern as are supernumerary placodes developing within the mammary line. At E13.5 strong Tbx3 expression is confined to the 10 mammary buds in controls, whereas mutants (with 100% penetrance) exhibit strong Tbx3 expression in both normally positioned mammary buds and supernumerary buds along the mammary line and in the ectopic cervical zone. We generally found one or two Tbx3 expressing ectopic buds occurring within each E13.5 mammary line, and as many as 22 ectopic cervical buds in a single A3cre/CAGC8 embryo (Fig. 2O,P). Ectodermal Wnt10b (Fig. 2Q,R, Supplemental Fig. 2E,F) shows a pattern of dysregulation remarkably similar to that of Tbx3 and Bmp2 in E11.5 mutant embryos. Because A3cre/CAGC8 embryos die at or around E14.5, later stages of mutant mammary development cannot be examined without orthotopic transplantation.

Somitic Hoxc8 misexpression results in supernumerary placode formation within the mammary line

We next induced Hoxc8 misexpression with Pax3cre (Engleka et al., 2005) in order to determine whether ectopic somitic Hoxc8 is sufficient to generate an anterior mammary permissive zone. In E11.5 Pax3cre/LacZ embryos, X-gal marks dorsal neural tube and strongly labels somites and hypaxial dermomyotome (Fig. 3A,B; n=2). Importantly, LacZ is not expressed in body surface ectoderm (Fig. 3B, inset) allowing us to test the competence of Hoxc8 in establishing an ectopic mammary permissive zone via its expression within somitic derivatives only. Like A3cre/CAGC8 embryos, Pax3cre/CAGC8 embryos died at or around E14.5. By E14.0 well formed ribs were established on all cervical vertebrae and the first three lumbar vertebrae of Pax3cre/CAGC8 embryos, whereas embryos carrying only the Pax3cre allele produced the wild type rib formula (Fig. 3C,D; n=6). Transformation of cervical vertebrae to a thoracic identity was accompanied by upregulation of the mammary factors Fgf10 and Tbx3 in hypaxial extensions of Pax3cre/CAGC8 cervical somites (Fig 3E-H; n=3 per genotype per probe). However, in contrast to A3cre/CAGC8 mutants, neither Tbx3 nor Bmp2 (n=2) expression showed focal upregulation in the cervical ectoderm of Pax3cre/CAGC8 mutants (Fig. 3G-J). Wnt/β-catenin signaling was examined in E13.5 Pax3cre/CAGC8 mice using the TOPgal reporter (DasGupta and Fuchs, 1999). Consistent with the absence of Tbx3 and Bmp2 expression in cervical placodes, no focal spots of high TOPgal expression were found anterior to MR1 in Pax3cre/CAGC8 mutants at E13.5 (Fig. 3K,L; n=7), suggesting the absence of ectopic cervical MR formation in these mutants. These results indicate that although ectopic Hoxc8 within somites is sufficient to expand thoracic vertebral
identity into the cervical region, it is insufficient to expand the zone of permissive mammary forming ectoderm. On the other hand, four out of seven mutant embryos (57%) formed a single unilateral supernumerary mammary bud between MR3 and MR4 in addition to all normally positioned MRs (Fig. 3K,L). This location overlies the hypaxial domain of endogenous Hoxc8 expression (Fig. 1G), and suggests that an increased level of somitic Hoxc8 can promote mammary placode development as long as it underlies a region of ectoderm that is competent for mammary formation.

**Simultaneous Hoxc8 misexpression in somites and overlying ectoderm establishes a cervical zone of mammary ectoderm**

To test whether simultaneous Hoxc8 expression in ectoderm and somitic derivatives is sufficient to recapitulate the anterior zone of cervical mammary placodes found in A3cre/CAGC8 mutants, we misexpressed Hoxc8 using a Wnt6cre driver (Makki, 2010). Wnt6 is initially expressed in a broad band of ectoderm encompassing the mammary forming region, and becomes restricted to the developing mammary placodes (Veltmaat et al., 2004). Analysis of E10.5 W6cre/LacZ embryos showed Wnt6 lineage extending across most of the surface ectoderm prior to mammary line formation (Fig. 4A and inset; n=3), although Wnt6 lineage was considerably weaker within lateral mesoderm compared to Hoxa3 lineage. Dermomyotomal expression of Wnt6 is restricted to the dorsomedial lip (Ikeya and Takada, 1998). Consequently, hypaxial signal (which derives from dorsolateral dermomyotome) was not detectable in Wnt6 lineage of W6cre/LacZ control embryos (Fig. 4a), or in Wnt6 lineage of W6cre/CAGC8 mutants (as visualized by the iresEGFP reporter (Fig. 4B)), indicating that ectopic Hoxc8 is restricted to non-hypaxial somite in this conditional cross.

Unlike A3cre/CAGC8 and Pax3cre/CAGC8 embryos, W6cre/CAGC8 embryos survive until birth, but die perinatally. We found rudimentary or fully formed ectopic ribs on one or two cervical vertebrae of W6cre/CAGC8 mutants (Fig. 4C,D; n=6), suggesting that hypaxial Hoxc8 expression is not required for transformation of cervical somites towards a thoracic identity. Somitic Hoxc8 expression was accompanied by upregulation of Fgf10 expression in cervical somites by E10.5 (Fig. 4E,F; n=4).

Expression patterns and levels of Tbx3 were nearly equivalent between control and W6cre/CAGC8 littermates at E10.5 (Fig. 4G,H; n=4), similar to A3cre/CAGC8 embryos and littermates at this stage. By E11.5, Tbx3 expression was focally upregulated in cervical ectoderm of Wnt6cre/CAGC8 mutants with 100% penetrance, indicating the formation of ectopic placodes (Fig. 4I-L; n=4). Whereas Tbx3 expression is aberrantly maintained in the cervical mesoderm of A3cre/CAGC8 mutant embryos at E11.5 (Fig. 2K,L), Tbx3 expression in W6cre/CAGC8 cervical mesoderm is similar to controls at this stage (Fig. 4G-J). Within the wild type mammary line, Tbx3 expression in control embryos was confined to placodes by E11.5, whereas W6cre/CAGC8 embryos exhibited lingering expression along the mammary line, and considerably broader, more diffuse expression within the forming placodes themselves (Fig. 4I,J). By E13.5, Tbx3 expression was focally restricted to mammary buds in both controls and mutants (Fig. 4M,N; n=4). W6cre/CAGC8 mutants had fewer ectopic buds at E13.5 than A3cre/CAGC8 mutants, with an average of 1 ectopic bud in each mammary line and up to 8 extra buds anterior to MR1.

At E13.5, Tbx3 antibody labeled all mammary bud epithelium (including supernumerary mammary rudiments), the surrounding mammary mesenchyme, and scattered cells in underlying mesoderm (Fig. 5A,B; n=4). In serial sections of the
same E13.5 embryos, Hoxc8 antibody labeled mutant epidermis and mammary primordia, as well as scattered cells in the underlying mesoderm (Fig. 1A, 5C, D; n=4), but was only present in scattered mesoderm of controls. We verified the mammary identity of ectopic cervical placodes in E13.5 W6cre/CAGC8 embryos by labeling mammary mesenchyme with antibodies for estrogen receptor alpha (ERα, n=2) and androgen receptor (AR, n=3). All supernumerary as well as normally positioned mammary placodes expressed both markers in control and mutant embryos (Fig. 5 E-H and data not shown). We found that Tbx3 expression was aberrantly upregulated in the epithelium of E13.5 W6cre/CAGC8 vibrissae at E13.5 relative to controls (Fig. 4M,N and supplemental Fig. 3A,B), leading us to speculate that vibrissal placodes may be adopting a mammary fate. However, neither ERα nor AR antibody labeled mesenchyme of the vibrissal placodes of control or W6cre/CAGC8 embryos (Supplemental Fig. 3C,D and data not shown, n=3) indicating that although vibrissal structures are incorrectly specified, ectopic Hoxc8 and consequent Tbx3 misexpression does not direct vibrissal differentiation towards a mammary program.

**Wnt/β-catenin signaling is abnormally upregulated in mammary placode epithelium of W6cre/CAGC8 embryos**

As Wnt signaling is essential for initiation and subsequent development of all ectodermal organs (Boras-Granic and Hamel, 2013; Chu et al., 2004; Lim and Nusse, 2013), we performed a detailed timeline of TOPgal reporter expression at different embryonic stages to study changes in Wnt signaling that accompany Hoxc8 dysregulation. W6cre/CAGC8 embryos survive until birth, enabling us to perform TOPgal analysis during mammary ductal elongation and branching. In E10.5 embryos, TOPgal reporter expression was consistently expanded in W6cre/CAGC8 cervical lateral mesoderm relative to control littermates (Fig. 6A,B; n=6), with expression extending rostrally to the 4th branchial arch. Limb and mammary line ectoderm showed similar faint staining between mutants and controls (Fig. 6A,B). Interestingly, the cervical pattern of Wnt/β-catenin signaling at E10.5 completely overlapped with Tbx3 expression at the same embryonic stage (Fig. 4G,H). At E11.5, the cervical TOPgal signal became more localized to the neck-forelimb junction. Vibratome sections through this region show considerably stronger signal in the mutant ectoderm and mesoderm compared to controls (Fig. 6C,D; n=4). Placode patterning along the wild type mammary lines of E11.5 control embryos appeared as focal aggregations of X-gal positive cells. However, Wnt signaling in the mutant showed ostensibly delayed aggregation of X-gal positive cells into mammary placodes. (Fig. 6C,D). Placode aggregation of Wnt10b expressing cells in W6cre/CAGC8 embryos at E11.5 paralleled the delay seen with TOPgal expression. (Supplemental Fig. 4, n=2).

At E12.5, both mutant and control embryos displayed diffuse patches of X-gal stained mesoderm caudal to the ear and in the cervical region. However, in the mutant, these diffuse stained patches contained focal spots stained dark blue, representing ectopic mammary primordia (Fig 6E,F; n=6), which resolve by E13.5 into three or four ectopic mammary buds anterior to MR1 on both sides (Fig. 6G-J; n=8). At E13.5, these ectopic buds often protruded abnormally from the ectoderm (Fig. 6H, top inset), but by E15.5 have invaginated into the underlying dermis as do MRs along the mammalian line (Fig. 6K,L,Q, R; n=3). Approximately 60% of E12.5 and E13.5 W6cre/CAGC8C8 mutant mice bore one or two small supernumerary mammary buds located between MR3 and MR4 (Fig. 6E,F). Normally positioned mammary buds of E12.5 and E13.5 W6cre/CAGC8 mutants always appeared larger than those of control littermates (as opposed to the ectopic buds, which were usually
smaller than endogenous buds), and by E13.5, MR3 was situated proximally and fused to MR2 in nearly half of all mutant embryos (Fig. 6H). Supernumerary, fused, and normally positioned mammary buds all expressed the downstream Wnt transcription factor, Lef1 (n=3) in mammary epithelium (Fig. 6G,H insets and data not shown).

By E15.5, Wnt/β-catenin signaling in controls is downregulated in the neck and surface epithelium overlying the growing mammary sprout. However, mammary sprouts of female W6cre/CAGC8 embryos often failed to downregulate Wnt/β-catenin signaling properly, particularly in the proximal part of MRs 2 and 3 that had fused or developed in close proximity. (Fig. 6K,L). In X-gal preparations of E17.5 whole skin, mammary rudiments of control embryos could be seen growing into the underlying secondary mammary mesenchyme (mammary fat pad) and branching into a primitive ductal tree. The ductal systems of all examined W6cre/CAGC8 mammary rudiments failed to develop substantially beyond the mammary sprout stage (Fig. 6M,N; n=3), and appeared to degenerate by birth. However, both control and mutant females were born with external nipples at anterior-posterior positions that corresponded to embryonic MR formation (Fig. 6O,P; n=3). In newborn controls, small mammary trees were associated with nipples, whereas mutant nipples were not associated with secondary mammary mesenchyme and lacked underlying ductal branches. Interestingly, nipples of mutant newborns maintained epithelial TOPgal signal and often had hair follicles associated with nipple epithelium (Fig. 6O,P). Moreover, both female and male W6cre/CAGC8 mutants maintained mammary sprout development after E14.5 within the cervical region (Fig. 6Q,R; n=3 each: females, males). In contrast, all MRs along the mammary line underwent regression at E14.5 in mutant males (n=3), as is normal in wild type male embryos (data not shown). Pelage hair placode and follicle morphology was normal in W6cre/CAGC8 mutant embryos (unpublished observations, Carroll and Capecchi). However, mutant vibrissal and whisker morphology was defective and TOPgal expression was aberrantly upregulated in vibrissal placodes by E12.5 (Fig. 6E-H, S,T).

Ectodermal Tbx3 ablation abolishes both ectopic and normal mammary rudiment formation

To determine the requirement of ectodermal Tbx3 in the formation of the anterior ectopic mammary zone, we misexpressed Hoxc8 while simultaneously ablating Tbx3 expression in the Wnt6 domain. Wnt6 lineage is strongly expressed in ectoderm, but is excluded from hypaxial dermomyotome, with restricted expression in lateral mesoderm prior to mammary placode formation (Fig. 4A,B). Consequently, all embryos with conditional ablation of one or both Tbx3 alleles maintained strong Tbx3 expression in hypaxial and lateral mesoderm, whereas no evidence of ectodermal Tbx3 expression was found in E11.5 W6cre/Tbx3Δ/Δ or W6cre/CAGC8/Tbx3Δ/Δ embryos (Fig. 7A-D; n>4 each genotype). One functional copy of ectodermal Tbx3 was sufficient to induce mammary rudiment formation in W6cre/Tbx3Δ/+ and W6cre/CAGC8/Tbx3Δ/+ embryos, with cervicalized localized rudiments forming (with 100% penetrance) in the latter (Fig. 7C,D). Wnt/β-catenin signaling was examined in W6cre/CAGC8 E13.5 embryos with one or both Tbx3 alleles conditionally deleted. TOPgal expression was present in cervical mammary buds of all W6cre/CAGC8/Tbx3Δ+/TOPgal embryos. (Fig. 7E,F; n=7), but was lost in all W6cre/CAGC8/Tbx3ΔΔ/TOPgal embryos (Fig. 7G; n=6). This establishes an ectoderm specific requirement of Tbx3 for mammary potentiation along the entire antero-posterior axis. In contrast to mammary rudiments, facial vibrissae and
whisker pads were maintained in the absence of ectodermal Tbx3 (Fig. 7E-G), affirming the divergent developmental trajectories of these two ectodermal appendages in response to ectopic Hoxc8.

We performed Chromatin immunoprecipitation (ChIP) on control E11.5 embryos to determine whether Hoxc8 is capable of direct transcriptional regulation of the Tbx3 promoter. A single primer set, located 1.5 kb 5’ of the Tbx3 ATG start codon, amplified Hoxc8-bound chromatin from both dorsal tissue (somites and neural tube) and ventrolateral thoracic tissue (mammary line ectoderm and mesoderm), but failed to amplify IgG-immunoprecipitated chromatin from either (supplementary methods, Supplemental Fig. 5). The experiment was successfully repeated on equivalent tissues derived from E11.5 W6cre/CAGC8 embryos, suggesting that Tbx3 may be directly regulated by Hoxc8 during mammary development of both control and mutant animals.

DISCUSSION

Somitic Hoxc8 misexpression instigates a rib-forming program and upregulates somitic mammary factor, Fgf10

We used a gene targeting approach to respecify thoracic identity in somites and surface ectoderm in order to test the competence of Hoxc8 regulated factors to potentiate mammary ectoderm and initiate placode formation. Expansion of the thoracic boundary was apparent in all of our mutant crosses by the appearance of ectopic ribs on cervical and other vertebrae. Accompanying the expansion of thoracic identity, we observed the consistent upregulation of somitic Fgf10 by ectopic Hoxc8 in all three of our conditional mutant crosses. Fgf10 expression, emanating from central (and possibly hypaxial) somites is one of the earliest and most critical regulators of mammary line initiation. Interestingly, a recent genome-wide association study found that variance in teat number in pigs was significantly associated with quantitative trait loci containing genes involved in vertebral development and possibly back length (Duijvesteijn et al., 2014). This raises the possibility that a Hoxc8 induced transformation of cervical into thoracic vertebrae creates a new signaling zone for mammary gland induction. However, ectopic Hoxc8 expression in Pax3cre/CAGC8 mutants produced completely penetrant cervical ribs without accompanying placode development in the cervical region. As these mutants often developed an additional MR along the normal mammary line, this suggests that somitic Hoxc8 misexpression (even without accompanying ectodermal expression) produces alterations in signaling gradients that can be interpreted by an ectoderm that is already specified for mammary development, but that somitic factors expressed during the thoracic patterning program cannot independently potentiate mammary ectoderm in the cervical region.

Ectopic Hoxc8 dysregulates mammary line Wnt signaling and placode patterning along the mammary line

Regulation of Wnt signaling by Hox factors has not been widely reported. However, as studies continue to uncover downstream targets of Hox genes, it is becoming apparent that this regulatory role of Hox genes has been overlooked. For example, using ChIP-seq, Donaldson et al. (2012) identified regions of the genome bound by Hoxa2 in the context of second branchial arch development. Of the thousands of
genes identified, the majority fell within the Gene Ontology (GO) category of ‘Wnt receptor signaling’. W6cre/CAGC8 mutants show mammary phenotypes with striking similarities to mice carrying targeted mutations of Wnt pathway modulators, Sostdc1 (Wise/Ectodin) and Lrp4 (Ahn et al., 2013; Närhi et al., 2012). Shared features between W6cre/CAGC8, Sostdc1−/− and Lrp4 null mice are: delayed downregulation of Wnt signaling within mammary epithelium, larger diameter of mammary placodes, supernumerary embryonic mammary rudiments developing along the mammary line (however, Sostdc1−/− mutant nipples only appear at puberty), and increased proximity and occasional fusion of mammary buds 2 and 3, which often protrude abnormally from the ectoderm. In addition, loss of Sostdc1 results in ectopic hair follicles developing within nipple tissue (Närhi et al., 2012). Ablation of the mesodermal mammary factor Gli3 in mice also shows notable similarities to the W6cre/CAGC8 mammary phenotype, likely due to dysregulation of Wnt signaling and of the crosstalk between Shh and Wnt pathways during mammary development (Hatsell and Cowin, 2006). Deletion of Gli3 causes inappropriate encroachment of hair follicles close to MR2, which itself protrudes abnormally from Gli3 mutant ectoderm, similar to cervical mammary placodes in W6cre/CAGC8 mutants. Interestingly, deletion of Gli3 also prevents the normal regression of mammary buds in male mice, comparable to the persistence of male mammary rudiments within the anterior ectopic zone of W6cre/CAGC8 mice (Chandramouli et al., 2013; Hatsell and Cowin, 2006; Lee et al., 2011; Lee et al., 2013; Ulloa et al., 2007).

Supernumerary mammary placode development has previously been reported in experimental mice, but always within or near the wild type mammary line, never in unique regions (Ahn et al., 2013; Chu et al., 2004; Howard et al., 2005; Mustonen et al., 2003). This difference between our and previous mouse models of mammary induction underscores the ability of ectopic Hoxc8 to initiate a mammary program, thus altering mesenchymal/ectodermal communication at the earliest stages of mammary line potentiation. Hoxc8 misexpression in both somites and overlying ectoderm enables the generation of a novel mammary zone followed by appropriate specification and early development of mammary rudiments up to the stage of ductal tree formation.

**A model of Hoxc8-induced ectopic mammary development**

A simplified model describing the role of ectopic Hoxc8 in potentiation of cervical mammary ectoderm is as follows (Supplemental Fig. 5): Somitic Hoxc8 expression upregulates somitic Fgf10 expression, which is a likely early requirement for potentiation of the cervical mammary zone, similar to the requirement of somitic Fgf10 for potentiation of the mammary line. However, somitic Hoxc8 expression alone is insufficient to induce cervical placode development (as evidenced by the lack of cervical MRs in the Pax3cre/CAGC8 phenotype), indicating an additional requirement for Hoxc8 expression in overlying ectoderm (as evidenced by robust cervical MR development in the W6cre/CAGC8 phenotype). Ectodermal Hoxc8 expression triggers upregulation of ectodermal Tbx3 expression, possibly via direct transcriptional activation, but only in specific regions where Tbx3 expression and Wnt signaling co-occur in underlying mesoderm. Ectodermal Tbx3 expression maintains Wnt signaling critical for ectodermal mammary potentiation. Within this ectopic cervical zone, Tbx3 expressing cells migrate towards placode positions based on somitic signaling gradients of Fgf10 and levels of Wnt activators.

This model is consistent with the absence of mammary programs initiated in other regions of mutant ectoderm that express Hoxc8 but lack either or both Wnt
signaling and *Tbx3* in underlying mesoderm. This model also predicts other regions of the W6cre/CAGC8 embryo in which ectodermal *Tbx3* upregulation was observed in association with Wnt signaling and *Hoxc8* expression, such as the whisker placodes, outer ear epidermis, and eyelid conjunctiva (Carroll and Capecchi, unpublished observations). These structures all lie in regions beyond somitic signaling gradients, and although they all were defective, none exhibited evidence of mammary rudiment development.

**Hox genes and normal mammary placode development**

Endogenous *Hoxc8* expression in E10.5 surface ectoderm is consistent with a scenario in which *Hoxc8* helps coordinate induction of mammary line ectoderm, but must be downregulated as epithelial cells migrate into proper position with respect to signaling gradients of *Fgf10* expression, and to *Tbx3, Gli3*, and other modulating factors that fine-tune levels of Wnt signaling. Following mammary line potentiation, somitic *Hoxc8* is well positioned to regulate specification of the third mammary placode, which develops in ectoderm directly overlying hypaxial extensions of somites 15 and 16.

Neither the *Hoxc8* knockout, nor Hox8 paralog knockout mice have reported mammary defects (Le Mouellic et al., 1992; van den Akker et al., 2001), although skin appendages were not specifically investigated in these mutants. Nevertheless, the scarcity of skin and skin accessory organ phenotypes exhibited by *Hox* deletion mutants is likely due to genetic compensation (Rossi et al., 2015), particularly functional rescue by other members of the *Hox* family of transcription factors, many of which exhibit overlapping expression patterns in fetal and adult skin (Boucherat et al., 2013; Chen and Capecchi, 1999; Welik and Capecchi, 2003). For this reason the complimentary approach of conditional misexpression/overexpression can be essential to unraveling developmental mechanisms that involve complex transcriptional programs and signaling pathways mediated by *Hox* genes and members of other large gene family networks. We suspect that myriad combinations of *Hox* transcription factors involved in placode patterning may provide the source of copious regional flexibility of cutaneous accessory organs that we see within and across taxa. However, it remains to be determined if other Hox genes have early transient activation within relevant ectodermal domains.

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Fig. 1.  **Hoxc8 lineage and expression.** (A) *Hoxc8* lineage of an E12.5 C8cre/YFP embryo includes all mammary buds (numbered arrows). The first mammary rudiment is obscured by the forelimb. (B) 10 μm sagittal sections through mammary rudiments (MR) 1, 2, and 5 of E13.5 wild type embryos labeled with Hoxc8 antibody (black nuclear stain). (C, D) Embryos in (C) and (D) are sectioned approximately along the planes indicated in the cartoon. (C) *Hoxc8* lineage (X-gal signal) in ventrolateral surface ectoderm. (D) Hoxc8 antibody (red) labels E10.5 ventrolateral surface ectoderm extending between and including both limb buds. (E) Hoxc8 is nearly absent from mammary placodes by E11.5, and from the surface ectoderm by E12.5 (ectodermal/mesenchymal boundary indicated by dotted line) (F). (G) *Hoxc8* expressing somites and hypaxial extensions in a wild type E11.5 embryo. Abbreviations: FL, forelimb; HL, hindlimb; MR, mammary rudiment; NT, neural tube; S, somite. Scale bars: 1mm (A,G); 500μm (C,D), 100 μm (B,E,F).
Fig. 2. Mammary placode markers are ectopically expressed in the expanded thoracic region of A3cre/CAGC8 embryos. (A) Hoxa3 lineage in an A3cre/LacZ E10.5 embryo. Inset is a 70 μm vibratome section through the region indicated by the dotted line. (B) Hoxa3 lineage in an E11.5 A3cre/CAGC8(IresEGFP) embryo marks the domain of Hoxc8 misexpression. (C) Control and (D) A3cre/CAGC8 mutant E14.5 skeletal preps. Red brackets indicate the formation of ectopic ribs. (E,F) Expression of Myf5 in E11.5 control (E) and A3cre/CAGC8 (F) embryos. Red brackets show ectopic hypaxial expression. (G,H) Bmp2 expression in control (G) and mutant (H) E11.5 embryos. (I,J) Fgf10 expression in E10.5 control (I) and A3cre/CAGC8 (J) embryos. Arrowheads in inset (J) show upregulated expression in cervical somites. (K,L) Tbx3 expression in E10.5 control (K) and A3cre/CAGC8 (L) embryos. Brackets indicate Tbx3 signal in lateral cervical mesoderm and pharyngeal arches, (drg) dorsal root ganglia, (o) otic vesicle. (M,N) Tbx3 expression in E11.5 control (M) and A3cre/CAGC8 (N) embryos. Brackets indicate anterior extension of lateral mesoderm (part of forelimb is damaged in N). (O,P) Tbx3 expression in E13.5 mammary buds of control and A3cre/CAGC8 embryos. (Q,R) Wnt10b expression in placodes and mammary line of E11.5 control (Q) and A3cre/CAGC8 (R) embryos. Dotted ovals indicate cervical mammary placodes/buds. Scale bars: 2mm (C,D); 1mm all other panels.
Fig. 3. Pax3cre/CAGC8 mutants have expanded thoracic identity, but do not develop cervical placodes. (A) Pax3 lineage in a whole mount E11.5 Pax3cre/LacZ embryo. (B) 12 μm paraffin cross-section of the X-gal stained embryo shown in (A) at level indicated by dotted line in (A). Arrowheads in insert show lack of signal in surface ectoderm. (d) dorsal, (v) ventral, (s) somite. (C) control and (D) Pax3cre/CAGC8 mutant E14 skeletal preps. Red brackets show ectopic ribs. (E,F) Fgf10 expression in E11.5 (E) control and (F) Pax3cre/CAGC8 embryos. Arrowheads in (F) inset show upregulated expression in central somites and hypaxial extensions. (G,H) Tbx3 expression in E11.5 (G) control and (H) Pax3cre/CAGC8 embryos. Arrowheads show ectopic expression in hypaxial extensions of cervical somites. (I,J) Bmp2 expression in E11.25 control (I) and Pax3cre/CAGC8 (J) embryos. (K,L) Frontal views of whole mount E13.5 TOPgal control (K) and TOPgal/Pax3cre/CAGC8 (L) embryos stained with X-gal. Heads and forelimbs are removed for clarity. The arrow points to a single supernumerary mammary bud (e). Scale bars: 1mm (A,E-L); 500μm (B); 2mm (C,D).
Fig. 4. Supernumerary mammary rudiments develop in cervical and mammary line ectoderm of W6cre/CAGC8 embryos. (A) Wnt6 lineage in an E10.5 W6cre/RosaLacZ embryo, (s) somites, (o) otic vesicle, (d) dorsal, (v) ventral. Inset shows a 70 μm vibratome cross-section through the region demarcated by the dotted line. Three bubbles (b) are trapped in this section. (B) Wnt6 lineage in an E10.5 W6cre/CAGC8(IresEGFP) embryo identifies the domain of Hoxc8 misexpression. Brackets indicate lack of Wnt6 lineage in hypaxial extensions. (C,D) Newborn control (C) and W6cre/CAGC8 (D) embryos showing mutant cervical ribs (arrowhead). (E,F) Fgf10 expression in E10.5 control (E) and W6cre/CAGC8 (F) embryos. Arrowheads in (F) inset show elevated expression in mutant cervical somites. (G,H) Tbx3 expression in E10.5 control (G) and W6cre/CAGC8 (H) embryos. Brackets indicate signal in cervical and pharyngeal arch mesoderm. (I,J) Tbx3 expression in E11.5 control (I) and W6cre/CAGC8 (J) embryos. Arrowheads show focal upregulation in cervical ectoderm. (K,L) 70 μm vibratome cross-sections through the regions indicated by dotted lines in (IJ). Red dotted lines demarcate the mesodermal-ectodermal border. (M,N) Tbx3 expression in E13.5 control (M) and W6cre/CAGC8 (N) embryos. Black arrowheads in N indicate ectopic cervical mammary rudiments. A supernumerary rudiment has formed along the mammary line (e, arrow). Red open arrowheads indicate accessory vibrissal placodes with aberrant Tbx3 signal. Scale bars: 1mm (A,B,E-J, M,N); 2mm (C,D); 100μm (K,L).
Fig. 5. Ectopic cervical rudiments express mammary specific markers
Parasagittal sections through the cervical/axillary region of E13.5 control (A,C,E,G) and W6cre/CAGC8 (B,D,F,H) embryos, labeled with Tbx3 (A,B), Hoxc8 (C,D), and mammary specific mesenchyme markers ERα (E,F), and AR (G,H). Ectopic cervical rudiments anterior to MR1 are indicated by ‘e’. Panels A-D show black antibody signal counterstained with nuclear fast red. Panels E-H show brown (DAB) antibody signal counterstained with a three-second hematoxylin dip. Scale bars: 200μm
Fig. 6. Wnt/β-catenin signaling during mammary rudiment development. (A,B) Brackets show upregulated TOPgal signal in the cervical region of E10.5 W6cre/CAGC8 embryos (B) compared to controls (A). Insets show faint signal along the mammary line. (C,D) An apparent delay of placode assembly (arrowheads) is observed in E11.5 W6cre/CAGC8 embryos (D) relative to controls (C). Insets show 70 μm vibratome cross-sections through equivalent levels within the boxed regions, (d) dorsal, (v) ventral. (E,F) TOPgal signal in E12.5 control (E) and W6cre/CAGC8 (F) embryos. Arrowheads show supernumerary mammary buds (G-J) TOPgal signal in E13.5 control (G,I) and W6cre/CAGC8 (H,J) embryos. Closed arrowheads in (H) point to an ectopic mammary bud ‘e’, and to the fusion between mutant MR2 and MR3. Frontal views in (I,J) show MR1 (arrows) and seven ectopic MRs anterior to MR1 in the mutant (arrowheads). Insets in G,H, and J show sections of E13.5 mammary buds labeled with antibody to Wnt target, Lef1. (K,L) TOPgal signal in 12 μm sections through E15.5 control (K) and W6cre/CAGC8 (L) mammary sprouts. Arrowhead identifies Wnt signal in ectoderm overlying mutant MRs 2 and 3 (arrows). The inset in (L) is a serial section from the same embryo. (M,N) TOPgal signal in E17.5 whole mount skin showing defective branching in the mutant (N) ductal tree. (O,P) 12 μm sections of control (O) and mutant (P) newborn nipples. Inset in (O) is a serial section through the same gland showing ductal branching. Closed and open arrowheads in (P) show defective Wnt/β-catenin signaling and hair follicle placement, respectively in nipple epithelium. (Q,R) TOPgal signal in 12 μm sagittal sections
through an ectopic mammary sprout in the cervical regions of E15.5 female (Q) and male (R) W6cre/CAGC8 embryos. (S,T) TOPgal signal in 12 μm sagittal sections though E15.5 control (S) and mutant (T) genal vibrissal follicles. All sections use nuclear fast red counterstain. Scale bars: 1mm (A-J main panels); 100μm (K-T and insets in G,H).
Fig. 7 Ectodermal ablation of *Tbx3* eliminates all mammary rudiments in *W6cre/CAGC8* embryos. (A-D) *Tbx3* expression in E11.5 control (A,C) and *W6cre/CAGC8* (B,D) embryos after conditional ablation of ectodermal *Tbx3*. Accompanying panels are 70 μm vibratome sections at the approximate sectioning planes shown in the pictured embryos. Dotted and solid lines in insets demarcate mesodermal and ectodermal boundaries respectively. Mammary placodes form in control (C) and mutant (D) embryos when only a single *Tbx3* allele is conditionally ablated (arrowheads). (E-G) TOPgal expression in E13.5 control (E), *W6cre/CAGC8/Tbx3Δ/+*TOPgal (F) and *W6cre/CAGC8/Tbx3Δ/Δ* /TOPgal (G) embryos. Accompanying panels are frontal views of the cervical region. White arrows point to the normal position of MR1. Arrowheads point to cervical mammary rudiments anterior to MR1. Loss of ectodermal *Tbx3* prevents MR formation (G) and leads to severe limb defects (arrow). Scale bars: 500μm (vibratome sections in A-D); 1mm (all other panels/insets).