

The T-box Transcription Factors TBX2 and TBX3 in Mammary Gland Development and Breast Cancer

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Abstract *TBX2* and *TBX3*, closely related members of the T-box family of transcription factor genes, are expressed in mammary tissue in both humans and mice. Ulnar mammary syndrome (UMS), an autosomal dominant disorder caused by mutations in *TBX3*, underscores the importance of *TBX3* in human breast development, while abnormal mammary gland development in *Tbx2* or *Tbx3* mutant mice provides models for experimental investigation. In addition to their roles in mammary development, aberrant expression of *TBX2* and *TBX3* is associated with breast cancer. *TBX2* is preferentially amplified in *BRCAl/2*-associated breast cancers and *TBX3* overexpression has been associated with advanced stage disease and estrogen-receptor-positive breast tumors. The regulation of *Tbx2* and *Tbx3* and the downstream targets of these genes in development and disease are not as yet fully elucidated. However, it is clear that the two genes play unique, context-dependent roles both in mammary gland development and in mammary tumorigenesis.

Keywords *Tbx2* · *Tbx3* · Mammary development · Breast cancer · Transcription factor · T-box

Abbreviations

E	Embryonic day
EMT	Epithelial–mesenchymal transition
HDAC	Histone deacetylase
MAP	Mitogen-activated protein

MG	Mammary gland
MP	Mammary placode
RA	Retinoic acid
TBE	T-box binding element
UMS	Ulnar mammary syndrome

Introduction

The first connection of the T-box gene family with mammary gland development came with the discovery that ulnar mammary syndrome (UMS, MIM 181450) is caused by mutations in *TBX3*. UMS is a rare autosomal dominant disorder so named for defects in the ulna and the mammary glands. Mammary gland abnormalities affect both sexes and include hypoplastic or aplastic breasts and supernumerary, inverted, or absent nipples with an inability to lactate [1, 2]. Axillary apocrine gland, dental, cardiac and reproductive system abnormalities are additional clinical features of UMS [1–5]. Both *TBX3* and the closely related *TBX2* are overexpressed in mammary gland neoplasia and breast cancer cell lines, suggesting a role for these genes in tumorigenesis and cancer progression. Studies in mice highlight the distinct roles that *Tbx2* and *Tbx3* play in mammary gland development and have characterized their roles in mammary gland induction and maintenance [6, 7]. However, the involvement of these genes in postnatal, pregnant, lactating or involuting mammary glands has yet to be explored.

TBX2 and *TBX3* are members of the T-box family of transcription factor genes, which are defined by the evolutionarily conserved DNA binding domain, the T-box. *TBX2* and *TBX3* share 95 % identical amino acid residues in their T-box domains and contain a highly conserved, C-terminal transcription-repression domain [2, 8, 9]. Although *TBX2* is expressed in a wide variety of tissues including the adult breast [10], there are no human developmental syndromes as yet associated with *TBX2*. The *TBX3* expression profile

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includes the adult breasts, heart, and uterus [1, 11]. UMS is caused by mutations throughout the *TBX3* gene [1, 2, 5]. Characterization of the crystal structure of TBX3 bound to DNA supported the supposition that point mutations affecting the T-box domain impair DNA binding [8]. C-terminal TBX3 mutants associated with UMS have decreased ability to repress transcription and accelerated rates of protein decay [9, 12]. Although some mutations disrupting the T-box domain have been associated with more severe mammary gland defects in males [3], mutations usually result in a spectrum of clinical features with no clear correlation between the location of a mutation and the UMS phenotype.

Tbx2 and *Tbx3* in Mouse Mammary Development

Tbx2 and *Tbx3* are the only members of the T-box family known to be expressed in developing mammary glands. Like their human counterparts, *Tbx2* and *Tbx3* share over 90 % sequence identity in their T-box domains and both contain a functional C-terminal transcription-repression domain [6, 9, 12]. At embryonic day (E) 9.5, neither gene is expressed in the mesenchyme at the ventro-lateral body wall between the forelimb and hindlimb where the mammary line will form within 1–2 days. Expression is first detected at E10.5 as a continuous stripe in the mesenchyme underlying the mammary line, with *Tbx3* exhibiting a broader domain of expression than *Tbx2* (Fig. 1a, b). While *Tbx2* is expressed only in mesenchyme at E10.5 and E11.5 (Fig. 1a, c), *Tbx3* is also expressed in the epithelium of mammary placode (MP) 1 and MP3 at E10.5 and in all 5 pairs of placodes at E11.5 (Fig. 1b, d, arrowheads). At E12.5 and E13.5, all mammary buds express *Tbx3* as well as *Lef1*, a downstream target of Wnt signaling and an early marker of mammary differentiation [6, 7, 13, 14]. At E18.5, both *Tbx2* and *Tbx3* are expressed in mesenchyme underlying the nipple sheath [7], but only *Tbx3* is expressed in the epithelium of the branching mammary ducts. The overlapping, yet distinct expression patterns of *Tbx2* and *Tbx3* are maintained throughout gestation. *Tbx2* expression in adults has not as yet been described, but *Tbx3* is expressed in virgin, pregnant, lactating and involuting mammary glands [6, 15].

Effects of *Tbx2* and *Tbx3* Mutations on Mammary Gland Development

Homozygosity for a null mutation of *Tbx2* results in embryonic lethality at midgestation, presumably due to heart abnormalities [16], while homozygosity for a null mutation of *Tbx3* results in embryonic lethality from mid to late gestation due to yolk sac and heart abnormalities [6]. Because of UMS, mammary gland development has been studied

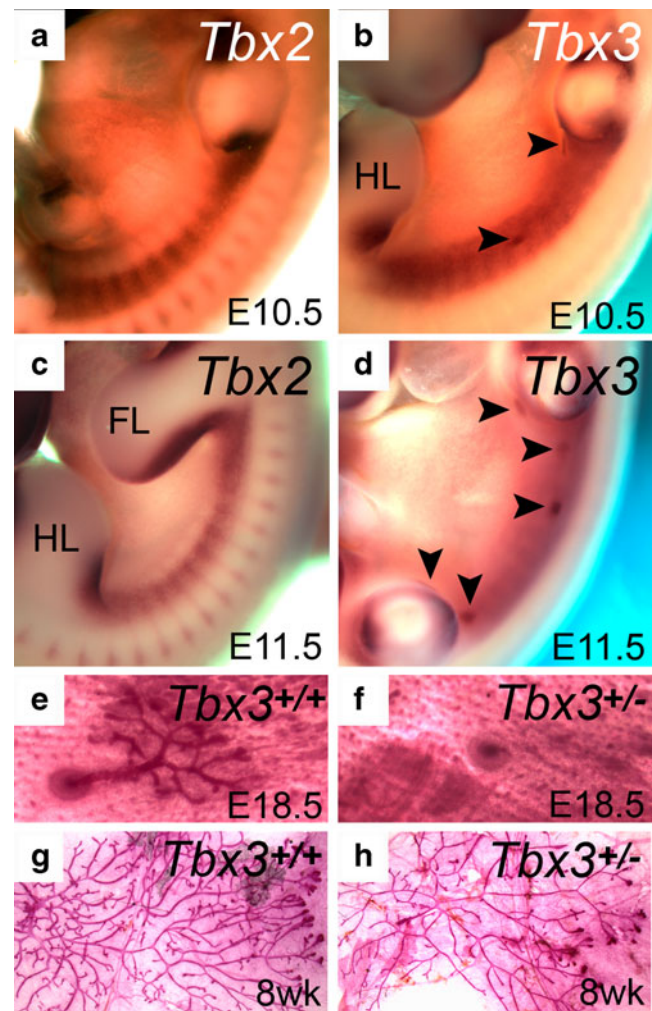


Fig. 1 Expression of *Tbx2* and *Tbx3* in the developing mammary gland and the effect of heterozygosity for a mutation in *Tbx3*. **a–d** Whole mount in situ hybridization for *Tbx2* and *Tbx3* at embryonic day (E) 10.5 and E11.5 shows expression of both genes in the mammary line between the forelimb and hindlimb and *Tbx3* expression in the mammary placodes when they first appear (arrowheads). **e, f** Skin and mammary gland preparation from late gestation embryos stained with Carmine's Alum to show the nipple and ductal tree, which is reduced in *Tbx3* heterozygotes. **g, h** Mammary gland preparations from 8 week old virgin females stained with Carmine's Alum showing a reduction in the branching network of *Tbx3* heterozygotes. (Modified with permission from [7])

extensively in *Tbx3* heterozygous and homozygous null mutants. At E13.5, expression of *Lef1* is similar in *Tbx3* heterozygous and wild type embryos, indicating normal mammary bud formation [7]. At E18.5, *Tbx3* heterozygotes have a significantly higher incidence of failed nipple and ductal tree development of mammary gland (MG) 1 and MG2 (Fig. 1e, f). When present, ductal trees in all mammary glands exhibit a trend towards fewer branches, with significantly fewer branches in MG1 and MG2 [7]. In *Tbx3* null homozygotes between E11.5 and E13.5, neither *Wnt10b* nor *Lef1* is expressed and at E12.5 there was no histological evidence of mammary placode formation in five mutant

embryos. By E13.5, however, histological examination revealed two rudimentary mammary buds in one mutant embryo and a single bud in a second embryo, both in the position of MG2 [6]. Adult virgin *Tbx3* heterozygous mice have a higher incidence of aplasia of MG1, MG2, and MG3 and reduced branching in ductal trees of all 5 pairs of mammary glands (Fig. 1g, h). Taken together, these data indicate that *Tbx3* is essential for normal mammary placode induction and that haploinsufficiency of *Tbx3* impairs mammary bud maintenance as well as the extent of ductal tree development.

Tbx2 homozygous mutants have no discernible defects in mammary placode induction [7]. Although all mammary glands are present in adult *Tbx2* heterozygous virgin females, the extent of ductal tree development is reduced, with a significant reduction in MG1. In *Tbx2*; *Tbx3* double heterozygous mice, there is a higher incidence of aplasia of MG1, MG2, and MG3 at E18.5 compared with *Tbx3* heterozygotes. These data reveal a genetic interaction between *Tbx2* and *Tbx3* in maintenance of mammary placodes [7].

Association with Human Mammary Neoplasia

TBX2 and *TBX3* are overexpressed in a number of cancers, including pancreatic, melanoma, and breast [10]. Amplification of the chromosomal region 17q22–q24, a region containing *TBX2*, *RPS6KB1* and several other candidate oncogenes, is common in breast cancers [17]. *TBX2* is preferentially amplified in *BRCA1/2*-associated tumors compared with sporadic tumors, whereas *RPS6KB1* is not [18, 19]. Furthermore, *TBX2* was amplified equivalently in ductal carcinoma in situ and invasive tumors, suggesting that *TBX2* amplification occurs early in the development of *BRCA1/2*-associated tumors [18]. In situ hybridization and Northern blot analyses confirmed that *TBX2* is overexpressed in primary tumors and breast cancer cell lines in which *TBX2* is amplified [17, 20]. *TBX3* is also overexpressed in a subset of breast cancer cell lines, with higher levels of *TBX3* in those with estrogen-receptor-positive status, such as MCF7, an epithelial adenocarcinoma cell line [11]. Treatment of MCF7 cells with estrogen induces *TBX3* expression, suggesting that estrogen increases the number of cancer stem-like cells in vivo breast tumors via *TBX3* signaling [21]. In a cohort of 79 breast cancer patients, *TBX3* expression was increased in plasma samples from all patients with advanced disease and in the majority of samples from the entire cohort [22]. *TBX3* protein is overexpressed in malignant breast epithelial cells compared with nonmalignant breast tissue [23]. Recent characterization of *TBX3* mutations in primary breast tumors strengthens the link between *TBX3* and mammary tumorigenesis [24, 25].

Molecular Mechanisms in Development and Neoplasia

Regulation of *Tbx2* and *Tbx3* in the Developing Mammary Gland

While more is known about the molecular function of *Tbx3* than *Tbx2* in mammary gland development, very little is known about how either gene is regulated in this tissue. In the MCF7 cell line, *TBX3* is upregulated in response to the phorbol ester PMA in a protein kinase C-dependent manner via the AP-1 factors c-Jun and JunB, which bind to an AP-1-response element in the *TBX3* promoter [26]. In early mammary gland development in vivo, induction of *Tbx3* expression depends on WNT signaling, retinoic acid (RA) signaling, and FGF signaling through *Fgfr2*. In the absence of *Tbx3*, *Wnt10b* and *Lef1*, RA signaling and FGF signaling are all lost [6, 14, 27], indicating feedback mechanisms in these three signaling pathways for induction and/or maintenance of *Tbx3* expression. In addition, overexpression studies have implicated BMP signaling in a reciprocal negative feedback loop with *Tbx3*, an interaction that may play a role in positioning the mammary placodes during development [28].

Downstream Targets and Mode of Action

Both *Tbx2* and *Tbx3* are transcriptional repressors and, by virtue of their highly similar DNA binding domains, can bind similar promoter sequences. However, their distinctive mutant phenotypes indicate that they do not function redundantly. In the mouse, overexpression of human *TBX3* in mammary epithelium causes hyperplasia by increasing proliferation and is associated with an increase in mammary stem-like cells. *TBX3* was shown to directly bind and repress NFκBIB, an inhibitor of the NFκB pathway known to play a role in regulating cell proliferation [29]. Combined with the hypoplastic phenotype seen in *Tbx3* mutants [6, 7], this indicates a proliferation-promoting role.

An important discovery was the direct transcriptional repression by both *TBX2* and *TBX3* of the cyclin dependent kinase inhibitors, *Cdkn2a* (*p19^{ARF}* in mouse; *p14^{ARF}* in humans), *p16^{INK4a}*, and *p21^{CIP}*, which are involved in proliferation control. *TBX2* was identified as a potent immortalizing gene in primary fibroblasts and *TBX3* was found to inhibit senescence in mouse immortalized neuronal cells [20, 30]. The senescence pathway is a protective mechanism against cancer and is mainly mediated by *p21^{CIP}*, *p16^{INK4a}* and *p19^{ARF}*, which stabilizes p53 levels. *TBX2* and *TBX3* regulate *p19/p14* through a variant T-box binding element (TBE) [31] and bind a consensus TBE in the *p21^{CIP}* promoter [32, 33]. There is evidence that *Tbx3* can promote the growth of mammary epithelial cells in vitro, with no effect on differentiation or apoptosis, through its ability to repress *p19^{ARF}* and *p21^{CIP}*, independently of the p53/Mdm2

pathway [15]. However, there is evidence that the hypoplastic mammary gland phenotype in heterozygous *Tbx3* mutants is independent of both p53 and p19^{ARF} [7].

The association of both *TBX2* and *TBX3* with mammary and other types of neoplasia has been a driver of discovery and in some cases mechanisms relevant to normal development have been uncovered in breast cancer cell lines. For example, interactions between *Fgf* and *Tbx3* signaling are involved in mammary gland initiation [14] and FGF9 expands the stem cell population in breast cancer cells and cell lines through FGFR signaling and *TBX3* [21]. However, whether other mechanisms identified in breast cancer cells apply to normal mammary gland development has yet to be determined. For example, *TBX3* physically interacts with histone deacetylases (HDACs) in MCF7 cells and its repressive function on *p14^{ARF}* is dependent on HDACs [23]. In the same cell line, it was found that in response to stress induced by UV irradiation, *Tbx2* protein is phosphorylated by p38 mitogen-activated protein (MAP) kinase, which leads to increased *Tbx2* protein levels, nuclear localization and an increase in the ability of *Tbx2* to repress the *p21* promoter [34]. Whether *TBX3* functions by recruiting HDACs to the TBE in the *p14^{ARF}* promoter and whether *Tbx2* repression of *p21* is enhanced in response to a stress-induced senescence pathway in development needs to be further explored.

Same or Different Functions?

A distinction between *TBX2* and *TBX3* function was highlighted using shRNA to knock down either *TBX2* or *TBX3* in MCF7 cells, which overexpress both genes. Knockdown of *TBX2* decreased proliferation without affecting migratory ability whereas knockdown of *TBX3* slightly increased proliferation and reduced migratory ability. At least in this context, *TBX2* acts as a powerful growth promoter, whereas *TBX3* is mainly required for cell migration [35]. The effect of *TBX3* on migratory behavior may underlie the increased invasiveness observed in response to the phorbol ester PMA since knockdown of *TBX3* abrogates PMA-induced cell motility in MCF7 cells [26]. The demonstration that E-cadherin is a downstream target of *TBX3* in invasive melanoma cells provides a possible mechanism for the effect on migratory behavior [36].

On the other hand, conflicting results were found with immortalized but otherwise normal mammary epithelial cell lines not expressing *TBX2* (mouse HC11 and human MCF10A). Expression of *TBX2* was sufficient to induce changes characteristic of epithelial–mesenchymal transition (EMT) with increased cell motility and invasion. *TBX2* was also upregulated upon induction of EMT in primary human mammary epithelial cells. Conversely, silencing endogenous

TBX2 in malignant human breast carcinoma cell lines MDA-MB-435 and MDA-MB-157 led to gain of epithelial characteristics, the loss of mesenchymal markers and the abolition of tumor cell invasion and metastatic potential. Like *TBX3*, *TBX2* was found to directly repress E-cadherin [37]. These conflicting results on whether or not *TBX2* promotes migratory cell behavior could be the result of cell type-dependent differences and highlight the need to consider the functions of these genes in specific contexts.

Conclusions

UMS highlights the role of *TBX3* in human mammary gland development, while studies in the mouse have shown that both *Tbx2* and *Tbx3* play important roles in the induction and maintenance of mammary placodes and in the growth of the mammary ductal tree. An important unexplored area is the role of these genes in the adult mammary gland during pregnancy, lactation and involution. *TBX2* and *TBX3* are overexpressed in primary breast cancers and breast cancer cell lines. The association of aberrant expression of both genes with human breast cancer has led to the idea that *TBX2* and/or *TBX3* could be used as prognostic biomarkers for breast cancer treatment [22].

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