Trp63 is regulated by STAT5 in mammary tissue and subject to differentiation in cancer

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Abstract

Transformation-related protein 63 (Trp63), the predominant member of the Trp53 family, contributes to epithelial differentiation and is expressed in breast neoplasia. Trp63 features two distinct promoters yielding specific mRNAs encoding two major TRP63 isoforms, a transactivating transcription factor and a dominant negative isoform. Specific TRP63 isoforms are linked to cell cycle arrest, apoptosis, survival, and epithelial mesenchymal transition (EMT). Although TRP63 overexpression in cultured cells is used to elucidate functions, little is known about Trp63 regulation in normal and cancerous mammary tissues. This study used ChIP-seq to interrogate transcription factor binding and histone modifications of the Trp63 locus in mammary tissue and RNA-seq and immunohistochemistry to gauge gene expression. H3K4me2 and H3K4me3 marks coincided only with the proximal promoter, supporting RNA-seq data showing the predominance of the dominant negative isoform. STAT5 bound specifically to the Trp63 proximal promoter and Trp63 mRNA levels were elevated upon deleting Stat5 from mammary tissue, suggesting its role as a negative regulator. The dominant negative TRP63 isoform was localized to nuclei of basal mammary epithelial cells throughout reproductive cycles and retained in a majority of the triple-negative cancers generated from loss of full-length Brca1. Increased expression of dominant negative isoforms was correlated with developmental windows of increased progesterone receptor binding to the proximal Trp63 promoter and decreased expression during lactation was correlated with STAT5 binding to the same region. TRP63 is present in the majority of triple-negative cancers resulting from loss of Brca1 but diminished in less differentiated cancer subtypes and in cancer cells undergoing EMT.
Introduction

The transformation-related protein 63 (Trp63) gene encodes two major isoforms, which are encoded by distinct mRNAs originating from two unique promoters (Yao & Chen 2012). The transactivating (TA) isoform carries an N-terminal acidic domain that is lacking in the more abundant dominant negative (DN) isoform. Both TA and DN transcripts undergo splicing events, generating additional isoforms, which differ at their COOH-terminus (Murray-Zmijewski et al. 2006). TRP63 along with TRP73 is a member of the TRP53 family of transcription factors. It binds and transactivates target genes of TRPS3 (Yang et al. 1998). TRP63 is required for epithelial tissue development, including mammary anlagen as well as limb and craniofacial development (Mills et al. 1999, Yang et al. 1999). TATrp63 isoforms are expressed before ΔNTrp63 isoforms during mouse embryogenesis (Yao & Chen 2012). High levels of TRP63 are found in basal cells of many tissues including mammary myoepithelial (Yang & McKeon 2000, Nylander et al. 2002, Sbisa et al. 2006, Truong et al. 2006, Forster et al. 2014, Yallowitz et al. 2014). Stem cells of epithelial tissues such as colon, urinary bladder, and prostate and mammary glands express TRP63 where it is thought to increase rates of apoptosis during the first phase of involution, pointing to a role for TRP63 in promoting MEC survival (Yallowitz et al. 2014). TRP63 has been shown to contribute to maintenance of parietal and regenerative abilities of the stem cell pool in prostate and mammary glands expressing TRP63 where it has been shown to contribute to maintenance of prototypical MECs (PI-MECs; Yallowitz et al. 2014). TRP63 has also been reported to regulate cell survival (Lifer et al. 2000, Senoo et al. 2004, Pietsch et al. 2008, Yallowitz et al. 2014), epithelial mesenchymal transition (EMT; Lindsay et al. 2011, Oh et al. 2011, Tran et al. 2013), and paracrine signaling in epidermis (Barton et al. 2010) and mammary gland (Forster et al. 2014). Different TRP63 isoforms have the ability, at least in overexpressing tissue culture cells, to regulate gene transcription and exhibit distinct and similar functions (Dohn et al. 2001, Koster et al. 2007). ΔNTrp63 isoforms have been reported to have longer half-life than TATrp63 isoforms (Yao & Chen 2012).

For a large percentage of target genes, ΔNTrp63 appears to be the primary regulator of expression, at least in epidermis (Barton et al. 2010). Consistent with this concept, in triple-negative breast cancer cell lines, both TA and DN isoforms have the ability to positively regulate caspase 1 and their co-expression is positively correlated with survival (Celardo et al. 2013). TA isoforms can prevent ΔNTrp63 isoforms from up-regulating expression of oncogenic miR155, expression of which is linked to tumor growth and migration (Mattiske et al. 2013). TATrp63 and its target Sharp1 inhibit metastasis of triple-negative breast cancer cells via degradation of hypoxia-induced factors (Montagnet et al. 2012, Piccolo et al. 2013). Expression of ΔNTrp63α inhibits EMT triggered by the ΔNTrp63γ isoform in normal mammary epithelial cells (MECs; Lindsay et al. 2011) and constrains EMT in bladder cancer cells (Oh et al. 2011). However, it has also been reported that ΔNTrp63α promotes EMT in normal keratinocytes (Tran et al. 2013). While it is known that TRP63 can be expressed in many types of cancer, the percentage of cancers within each type expressing TRP63 varies (Yao & Chen 2012). In mammary tissue, TRP63 has been shown to contribute to maintenance of parietal-identified MECs (PI-MECs; Yallowitz et al. 2014) and TRP63 haploinsufficiency reduces pregnancy-promoted ERBB2 tumorigenesis in transgenic mice expressing activated ERBB2 (Yallowitz et al. 2014).

Post-pubertal development of mammary tissue is typified by hormonally regulated cycles of epithelial cell proliferation, function and apoptosis during estrus (Fata et al. 2001), pregnancy (Li et al. 1997, Schorr et al. 1999), lactation, and involution. During pregnancy, the prolonged proliferative phase triggered by estrogen and progestosterone signaling is followed by relative quiescence with lactation, and apoptosis during involution correlated with phosphorylation and activation of STAT3. TRP63 in mammary myoepithelial cells is required for lobuloalveolar development during pregnancy (Forster et al. 2014) through controlling neuregulins 1 and 2 (Nrg1 and Nrg2) expression (Forster et al. 2014, Yallowitz et al. 2014). NRG1 is a paracrine factor expressed in mammary myoepithelial cells required for normal ERBB4 and STAT5 activation in luminal mammary epithelium (Forster et al. 2014). STAT5 activation in mammary luminal progenitor cells is required for normal lobuloalveolar development (Liu et al. 1997, Cui et al. 2004, Yamaaji et al. 2009). TRP63 also contributes to regulating involution. Germline loss of one Trp63 allele is sufficient to increase rates of apoptosis during the first phase of involution, pointing to a role for TRP63 in promoting MEC survival (Yallowitz et al. 2014). By contrast, apoptosis and involution proceed normally when both Trp53 alleles are absent from the germline (Li et al. 1996). STAT3 has been reported to activate ΔNTrp63α in Hep3B cells (Chu et al. 2008).
The presence of TRP63 is a proposed biomarker for basal cancer (Thike et al. 2010a, Shekhar et al. 2013) but not all investigations concur (Buckley et al. 2011, Thike et al. 2013). TRP63, cytokeratin 5 (KRT5), and smooth muscle actin (ACTA2) are expressed coordinately in normal basal mammary myoepithelial cells but are not invariably synchronously expressed in human breast cancers (Laakso et al. 2005, Jumppanen et al. 2007). Two different basal-like (BL) cancer subtypes are recognized, with BL2 but not BL1 demonstrating TRP63 expression with a poorer response to neoadjuvant chemotherapy as compared with BL1 (Lehmann et al. 2011, Masuda et al. 2013). In one study, higher TRP63 expression levels were positively correlated with brain metastasis (Shao et al. 2011). Positive staining for TRP63 can be used to differentiate intraductal papilloma from ductal carcinoma in situ (Moriya et al. 2009, Furuya et al. 2012). TRP63 is required for collective invasion of breast cancer organoids in 3D culture even if the cells used for culturing were acquired from primary tumors characterized as luminal rather than myoepithelial (Cheung et al. 2013).

Breast cancer susceptibility gene 1 (BRCA1) has been reported to increase transcription of the ΔNTRP63 isoforms in tissue culture cells (Buckley et al. 2011, Buckley & Mullan 2012), while in vivo the presence of Trp63 expression is correlated with decreased expression of BRCA1 in basal-type breast cancers (Ribeiro-Silva et al. 2005). BRCA1 and ΔNTRP63 also co-regulate expression of NOTCH signaling in mammary basal cells and loss of NOTCH would result in increased expression of stem/progenitor pool, loss of markers for terminal differentiation, and simultaneous increase in basal markers (Buckley & Mullan 2012). Women who carry BRCA1 mutations demonstrate an increase in the percentage of luminal progenitor cells, which are thought to represent precursor cells for BL triple-negative breast cancers (Lim et al. 2009).

Triple-negative breast cancers are over-represented in women who carry BRCA1 mutations as compared with women who develop breast cancer without BRCA1 mutation (Ribeiro-Silva et al. 2005, Liu et al. 2008). This predilection is modeled in genetically engineered mice with a MEC-specific deletion of full-length Brca1 (Hershkovitz et al. 2007) allowing us to explore the impact of loss of full-length Brca1 on p63 expression levels.

This study now addressed the critical void in our understanding of in vivo regulation of Trp63 in mammary gland development and cancer. Specific objectives were to assess the impact of BRCA1 and understand the relationship with cancer cell differentiation and EMT.

Materials and methods

Mouse models

Female C57Bl/6 parental inbred and litter-mate control WT, Brca1floxed exon 11 (f11)/f11/mouse mammary tumor virus (MMTV)-Cre/Trp53+/-, Brca1f11/WT/MMTV-Cre/Trp53+/-, Brca1f11/WT/MMTV-Cre/Trp53+/-, and Brca1WT/WT/MMTV-Cre/Trp53+/-/tet-op–CYP19A1/MMTV–rtTA mice were identified by PCR (Jones et al. 2005; Transsenta, Cordova, TN, USA), maintained in barrier zones in sterilized ventilated cages with corncob bedding and allowed to access water/chow ad libitum under 12 h light:12 h darkness cycles at Georgetown University (one to four mice per cage) or NIH (one mouse per cage). Mice were killed by CO2 narcosis and tissues were removed at necropsy following the guidelines approved by the GUACUC/NIHACUC. Mammary glands were isolated from 2.5-, 6-, 8-, 10-, 16-, and 24-week-old mice that were nulliparous, pregnant (P; days 6 and 17, calculated from the day of first appearance of vaginal plug), lactating (L; days 1 and 10), and undergoing involution (In; days 1, 3, and 7) (n = 6 nulliparous mice; n = 3 qRT-PCR/immunohistochemistry (IHC); n = 3, one mouse each estrus, proestrus, and diestrus; and n = 4 P, L, and In mice). Estrous cycle stages were identified by vaginal cytology. Mammary cancers were isolated from 10- to 12-month-old nulliparous Brca1f11/WT/MMTV-Cre/Trp53+/-, Brca1f11/WT/MMTV-Cre/Trp53+/-, and Brca1WT/WT/MMTV-Cre/Trp53+/-/tet-op–CYP19A1/MMTV–rtTA mice (n = 12). Dissected abdominal mammary glands and minimum of one-third of tissue/cancer isolated from surrounding normal tissue were flash frozen in liquid nitrogen and a second/third were fixed in 10% formalin and paraffin embedded.

Histology and IHC

Sections of 5 μm were stained with hematoxylin and eosin (H&E) or used for IHC: TRP63, MS-1081-P, 1:200; ACTA2, 1:500 (Epitomics, Burlingame, CA, USA); TATRP63, sc-8608, 1:750; Her2/neu, sc-284, 1:400 (Santa Cruz Biotechnology); single-stranded (ss) DNA, ALX-804-192, 1:10 (Alexis Biochemicals, Farmingdale, NY, USA); KRT5, sc-542, 1:750; PGR, sc-538, 1:750; Her2/neu, sc-284, 1:400 (Santa Cruz Biotechnology); single-stranded DNA, ALX-804-192, 1:10 (Alexis Biochemicals, Farmingdale, NY, USA); and KRT5, sc-542, 1:750; TATRP63, sc-8608, 1:40; ESR1, sc-542, 1:750; PGR, sc-538, 1:750; Her2/ne, sc-284, 1:400 (Santa Cruz Biotechnology); single-stranded (ss) DNA, ALX-804-192, 1:10 (Alexis Biochemicals, Farmingdale, NY, USA); and ACTA2, 1:500 (Epitomics, Burlingame, CA, USA).
antibody. Digital images were generated using a Nikon Eclipse E800 Microscope using the Nikon DMX1200 Software (Nikon Instruments, Inc., Melville, NY, USA).

Primary culture

Primary mammary epithelial cancer cells were isolated (EpiCult-B (mouse), STEMCELL Technologies, Vancouver, CA, USA), divided, and cultured either to maintain expression of epithelial differentiation markers (E) as conditionally reprogrammed cells (Liu et al. 2012) or under conditions promoting expression of genes mediating EMT (EpiCult-B). Then they were removed and placed into the standard Gibco DMEM with 10% fetal bovine serum and 1% Pen/Strep (Life Technologies). Cells were cultured at 37°C with 5% CO2 until they reached 85% confluency (48–72 h) and washed three times with 1× Gibco PBS (PBS) (Life Technologies). After washing, they were collected by scraping into 1.5 ml Eppendorf tubes, washed again with 1× PBS, sedimented by centrifugation at 1000 g for 5 min followed by washing three times with 1× PBS, and frozen in liquid nitrogen.

RNA isolation, cDNA synthesis, qRT-PCR, RT-PCR, RNA-seq, ChiP-seq, and statistical analyses

For qRT-PCR, RNA was extracted using the Invitrogen PureLink Micro-to-Midi RNA Extraction Kit #12183-018 and Qiagen Shredder Kit #79654. Integrity was confirmed by the presence of 18S and 28S bands after agarose gel electrophoresis, and first-strand cDNA was synthesized. Expression levels of different Trp63 isoforms were determined using Mm00495788_m1 for total Trp63 (inventoried assay) and custom designed assays (Applied Biosystems): SApSh3T-EDS for TA

...Trp63

ACTGTTCAGGGATCTT); and SApSh3G-EDDp for (F, GGGCTGACCACCATCTAT and R, GTCGGGA-

...Trp63

D

AACCTCGATGGGCTGTACTG); SApSh3D-SpC for CCCAGAGGTCTTCCAGCATATCT and reverse (R), Trp63

determined using Mm00495788_m1 for total Trp63

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AGGAGCCCCAGGTTCGT); SApSh3A-ESCR for Trp63

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mRNA

Immunoblotting

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Diego, CA, USA). The...Trp63

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Endocrine-Related Cancer cells (Fig. 2). This raises the possibility of STAT5 acting as a MECs while STAT5 and PGR are expressed at high levels in luminal PGR binding to the distal promoter (data not shown). Both nulliparous mice, weak H3K4me2 marks coincided with lactation (Fig. 1A); however, in primary MECs from distal, promoter coincided with H3K4me2 marks during of TA Trp63 D STAT5a/STAT5b, was above 10 FPKM at both P6 and L1. Trp63 regulator of genetic experiment indicated that STAT5 is a negative Stat5b allele, which leads to an By contrast, Trp63 expression amounted to 5 FPKM at both P6 and L1 (Fig. 1B). nulliparous. (B) Bar graphs illustrating relative fragments per kilobase of treated with E for 24 h then E diestrus; H3K4me2: liver, L8; and PGR: MG, ovariectomized nulliparous mice H3K4me3: MG, L1; H3K4me2: MECs, nulliparous–distal. (A) Available STAT5, PGR, H3K4me2, and H3K4me3 ChIP-seq data of mammary gland and liver were reanalyzed to unveil usage of proximal and distal Trp63 promoters. The seven tracks illustrated are (top to bottom): H3K4me3: MG, L1; H3K4me2: MG, L8; H3K4me2: MECs, nulliparous–diestrus; H3K4me2: liver, L8; and PGR. MG, ovariectomized nulliparous mice treated with E for 24 h then E + Pr for 6 h; STAT5a: MG, L1; and STAT5b: liver, nulliparous. To explore a functional role for STAT5, RNA-seq was employed to analyze Trp63 levels in the presence of different numbers of Stat5a/Stat5b alleles. In WT mammary tissue, which is carrying intact Stat5a and Stat5b alleles, Trp63 expression amounted to 5 FPKM at both P6 and L1 (Fig. 1B). By contrast, Trp63 expression in the presence of only one Stat5b allele, which leads to an ~90% reduction of STAT5a/STAT5b, was above 10 FPKM at both P6 and L1. Levels of ΔNTp63 isoforms were much higher than those of TATp63 isoforms as predicted by the ChIP-seq. This genetic experiment indicated that STAT5 is a negative regulator of Trp63. PGR binding at the proximal, but not distal, promoter coincided with H3K4me2 marks during lactation (Fig. 1A); however, in primary MECs from nulliparous mice, weak H3K4me2 marks coincided with PGR binding to the distal promoter (data not shown). Both STAT5 and PGR are expressed at high levels in luminal MECs while ΔNTRP63 is localized to basal myoepithelial cells (Fig. 2). This raises the possibility of STAT5 acting as a negative regulator of ΔNTRP63 in luminal cells.

TRP63 and ΔNTRP63 were localized to basal MEC nuclei throughout reproductive development

IHC for both TRP63 and ΔNTRP63 was performed in mammary tissue obtained from WT mice that were nulliparous, pregnant, lactating, or undergoing involution to determine if tissue or cellular localization changes during reproduction. KRT5 IHC was used as an independent marker for basal myoepithelial cells (Bankfalvi et al. 2004) and ssDNA IHC was performed to identify apoptotic cells (Frankfurt 2004). Basal myoepithelial cells were the most prominent cell type expressing TRP63 and ΔNTRP63 in all samples with nuclear localization throughout the cycle (Fig. 2). Both small (large panels) and large (insets) ducts and alveoli (large panels) were examined with localization of TRP63 and ΔNTRP63 to myoepithelial cells throughout. The relatively contiguous arrangement of basal myoepithelial cells expressing nuclear-localized TRP63 and ΔNTRP63 in mammary gland from nulliparous mice evolved to a more dispersed pattern in late pregnancy (p17) and lactation, returning to the pattern found in nulliparous mice by involution day 7 (In7). Myoepithelial cells appeared to undergo apoptosis during involution, defined by their location within tissue and the presence of TRP63, ΔNTRP63, and KRT5 staining in cells shed into the lumen. During the first 3 days of involution, apoptotic MECs are shed into the alveolar lumen (Schorr & Furth 2000). Experiments indicated that patterns of Trp63 and ΔNtrp63 expression measured in total mammary gland are primarily derived from basal myoepithelial cells at all stages of reproductive development.

Trp63 expression was regulated during mammary gland reproductive development

Real-time RT-PCR was used to characterize expression levels of ΔNtrp63, total Trp63, TATp63, Trp63α, and

Figure 1
Histone modification and transcription factor binding to the Trp63 locus. (A) Available STAT5, PGR, H3K4me2, and H3K4me3 ChIP-seq data of mammary gland and liver were reanalyzed to visualize usage of proximal and distal Trp63 promoters. The seven tracks illustrated are (top to bottom): H3K4me3: MG, L1; H3K4me2: MG, L8; H3K4me2: MECs, nulliparous–diestrus; H3K4me2: liver, L8; and PGR. MG, ovariectomized nulliparous mice treated with E for 24 h then E + Pr for 6 h; STAT5a: MG, L1; and STAT5b: liver, nulliparous. (B) Bar graphs illustrating relative fragments per kilobase of total transcript per million mapped (FPKM) reads of Trp63, TATp63, and ΔNTp63 in mammary glands from genetically engineered STAT5-deficient (STAT5a−/−, STAT5b−/−) (light gray) and WT (STAT5a−/−, STAT5b−/−) (dark gray) mice at P6 and L1 as estimated by RNA-seq. PGR, progesterone receptor; MG, mammary gland; MECs, mammary epithelial cells; GAS, γ-interferon-activated sequence; PRE, progesterone receptor-binding element; L1, lactation day 1; P6, pregnancy day 6; E, 17β-estradiol (100 ng); and Pr, progesterone (2.5 μg).
Figure 2
Immunohistochemical staining of distinct biological phases of post-pubertal mammary gland development and involution. Representative images illustrating expression patterns of total TRP63, ΔNTRP63, KRT5, and single-stranded (ss) DNA in mammary gland from nulliparous, pregnancy day 17 (P17), lactation day 1 (L1), and involution day 1 (In1) mice. Images shown in large panels are small ducts (nulliparous and In7) and alveoli (P17, L1, L10, In1, and In3). Images shown in insets are large ducts. TRP63, ΔNTRP63: arrows point to representative cells with nuclear-localized TRP63 and ΔNTRP63 expression. Closed arrowheads point to representative cells shed into the lumen demonstrating TRP63 and ΔNTRP63 expression. KRT5: open arrowheads point to representative cells with KRT5 expression. Closed arrowheads point to representative cells with myoepithelial cell localization with reactivity for ssDNA. Closed arrowheads point to representative cells shed into the lumen with reactivity for ssDNA. Large panels taken at 60× magnification, insets at 20× magnification. Scale bar = 10 μm.
Figure 3
Changes in expression levels of Trp63 isoforms during normal mammary gland development. (A) Structure of the Trp63 gene. Splicing (lines) and alternative promoters (arrows) are indicated. Locations of primers used for real-time RT-PCR (B) and RT-PCR (Fig. 5) are illustrated. (B) Box and whisker plots demonstrating changes in relative expression levels of ΔNTrp63, total Trp63, TA Trp63, Trp63α, and Trp63γ in mammary gland tissue from nulliparous, pregnancy day 17 (P17), lactation day 1 (L1), and involution day 1 (In1) mice are shown along with line graphs comparing the relative expression levels of ΔNTrp63 and TA Trp63 across these reproductive stages. Expression levels of Trp63 isoforms are normalized to 18S. *The earliest stage with a statistically significant drop in expression after the nulliparous stage (P<0.05; Mann–Whitney U test). #The earliest stage with a statistically significant increase in expression level after L10 (P<0.05; Mann–Whitney U test).
Trp63γ throughout the reproductive cycle (Fig. 3). For comparison, locations of the real-time RT-PCR primers used for detection of different spliced forms during reproduction and the RT-PCR primers used to analyze expression in cancers are both indicated herein along with exon structure, locations of the TATp63 and ΔNTrp63 promoters, and 3′-termini of the Trp63α and Trp63γ spliced forms (Fig. 3A). It was not possible to design primers to specifically detect the Trp63β spliced form. The higher sensitivity of the real-time RT-PCR compared with levels of sequencing used for the RNA-seq (Fig. 1B) enabled detection of both ΔNTrp63 and TATp63 spliced forms (Fig. 3B). Expression levels of both spliced forms were lowest at L10 but the kinetics of change differed. ΔNTrp63 expression was significantly lower at L1 (P < 0.05; Mann–Whitney U test compared with nulliparous, P17) while expression levels of TATp63 dropped significantly only at L10 (P < 0.05; Mann–Whitney U test compared with nulliparous, P17 and L1). Likely due to the overall higher expression levels of ΔNTrp63 compared with TATp63, the significant drop in total Trp63 occurred at L1 (P < 0.05; Mann–Whitney U test compared with nulliparous, P17) as shown specifically for ΔNTrp63. Trp63α forms also dropped significantly at L1 (P < 0.05; Mann–Whitney U test compared with nulliparous, P17) while Trp63γ forms showed a different pattern dropping significantly at P17 (P < 0.05; Mann–Whitney U test compared with nulliparous). Expression of ΔNTrp63, total Trp63, Trp63α, and Trp63γ all rose significantly at In1 (all P < 0.05; Mann–Whitney U test compared with L1) but again the pattern of TATp63 was unique, rising significantly only at In3. Line graphs indicated that expression of ΔNTrp63 was higher than that of TATp63 throughout reproduction. When studying gene expression during mammary gland development, interpretations of significant expression drops during lactation must include consideration of the high levels of milk protein RNAs produced at that time that could produce a dilution effect. While the highest levels of milk protein RNAs may be found at L10, milk protein RNA expression is present at L1 and In1. It is possible that lower levels of Trp63 expression were correlated with a change in cell type percentages in the mammary gland during lactation as milk-producing luminal cells were relatively more numerous than the more dispersed TRP63-expressing basal myoepithelial cells during lactation (Fig. 2). Finally, expression levels of phosphorylated STAT5, which appeared to act as a negative transcription factor (Fig. 1), are highest during lactation (Liu et al. 1996). RNA-seq data from WT nulliparous mice sampled during the estrous cycle demonstrated a doubling in Trp63 expression from 4 FPKM during estrus to 10 and 11 FPKM, respectively, during proestrus and diestrus, confirming regulated gene expression during the second type of reproductive cycle.

**Disruption of full-length Brca1 expression in mammary epithelium did not alter TRP63 expression patterns during mammary gland development**

Trp63 is required for establishment of mammary placodes (Yang et al. 1999). Brca1 is expressed in mammary epithelium before puberty (Marquis et al. 1995) and has been reported to positively regulate Trp63 expression (Buckley et al. 2011). To determine if loss of full-length Brca1 disrupted the pattern of TRP63 expression or myoepithelial cell differentiation, expression patterns of TRP63 and KRT5 determined by IHC were compared in mammary tissues from WT mice and Brca1-null mice with loss of full-length Brca1 targeted to MECs (Brca1f11/f11/MMTV-Cre/Trp53+/+ mice; Fig. 4). Expression patterns of TRP63 in Brca1-null mice resembled those found in WT mice, clearly detectable as nuclear-localized in basally located cells by 6 weeks of age and continuing without change at 10 weeks, 4 months, and 6 months of age. Cytoplasmically located KRT5 showed a similar pattern, also expressed at significant levels in basal myoepithelial cells by 6 weeks of age. The data indicated that full-length Brca1 does not play an essential role in regulating TRP63 expression during normal development in vivo.

**Expression levels of Trp63 in cancer were positively correlated with differentiation and intact Brca1**

Triple-negative mammary cancers develop in nulliparous mice with loss of full-length Brca1 targeted to MECs when one copy of germline Trp53 is disrupted (Jones et al. 2005). To assess Trp63 expression in mammary cancer cells in the absence of full-length Brca1, a set of ten triple-negative mammary carcinomas from Brca1f11/f11/MMTV-Cre/Trp53+/- mice were evaluated by RT-PCR and IHC (Fig. 5). The set included non-basal and basal subtypes defined by cDNA array analyses and included eight adenocarcinomas (1, 3, and 5–10) and two spindloid cancers (2 and 4) (Herschkowitz et al. 2007). In general, ΔNTrp63 spliced forms were expressed at higher levels than TATp63 spliced forms with the highest levels found in more differentiated adenocarcinomas with predominantly nuclear localization of TRP63 and ΔNTrp63 (Fig. 5). However, cytoplasmic location and additional Trp63 splice variants have also been reported in human cancers. Herein, the
spindloid cancers showed a different pattern with undetectable RNA expression in one correlated with cytoplasmic staining (2) and relatively equal TA and ΔNTrp63g expression levels in the other (4). It is possible that the cancer with cytoplasmic staining expressed an aberrant splice variant undetectable with the PCR primers used, or the staining pattern is somehow artifactual, even when appropriately controlled.

Brca1f11/f11/MMTV-Cre/Trp53/C/Km mice exhibit histological tumor heterogeneity (Nakles et al. 2013), which is the simplest explanation for disconnects in RNA and protein expression levels observed (6 and 9), but altered regulation of TRP63 translation cannot be excluded. KRT5 and ACTA2 are basal cell markers (Abd El-Rehim et al. 2004, Sarrio et al. 2008, Thike et al. 2010b). Protein expression localized to cancer cells was evaluated using IHC (Fig. 5C). Cancers classified as basal subtype demonstrating TRP63 also showed expression for KRT5 but not invariably ACTA2. KRT5 expression is not demonstrated in cancers classified as non-basal subtype with lower levels of TRP63 expression, while one of the cancers with higher levels of ΔNTrp63 expression demonstrated KRT5 expression localized to cells in a ring-like pattern surrounding KRT5-negative cells. Some cancers that did not demonstrate KRT5 expression showed ACTA2 expression. Primary mammary cancer cell lines derived from triple-negative adenocarcinomas with two intact Brca1 alleles (Brca1+/+Trp53+/−) from Brca1WT/WT/MMTV-Cre/Trp53+/−/tet-op–CYP19A1/MMTV–rtTA mice, one intact Brca1 allele (Brca1+/−Trp53+/−) from Brca1WT/WT/MMTV-Cre/Trp53+/−/tet-op–CYP19A1/MMTV–rtTA mice, and two disrupted Brca1 alleles (Brca1−/−Trp53+/−) from Brca1f11/f11/MMTV-Cre/Trp53−/− mice were used to test if loss of full-length Brca1 reduced Trp63 expression. Because Trp63 expression was higher in more differentiated cancers, the cell cultures were divided and cultured under two conditions, conditional reprogramming (Liu et al. 2012) that maintained epithelial cuboidal morphology (E) or EpiCult-B (STEMCELL Technologies), permissive for EMT, and then harvested for analysis of gene expression by RNA-seq. Significantly higher fold expression of MEC differentiation genes (Krt5, >2000-fold; Krt8, greater than twofold; Krt1 and Krt4, >40-fold; and Krt18, greater than twofold) was

Figure 4
Immunohistochemical staining of TRP63 and KRT5 in mammary tissue from mice with and without expression of full-length Brca1. Representative images illustrating expression patterns of total TRP63 and KRT5 in mammary gland from nulliparous Brca1-null (Brca1f11/f11/MMTV-Cre/Trp53+/−) and WT mice at ages 2.5, 6, and 10 weeks and 4 and 6 months. Images were taken at 40× magnification. Scale bar = 25 µm.
Figure 5
Expression levels of different Trp63 isoforms and basal markers in mammary carcinomas developing in Brca1<sup>+/−</sup>MMTV-Cre<sup>−/−</sup>Trp53<sup>−/−</sup> mice classified as either non-basal or basal. (A) Relative expression levels of TA<sub>Trp63α</sub> (2043 bp), TA<sub>Trp63β</sub> (1664 bp), TA<sub>Trp63γ</sub> (1407 bp), ΔN<sub>Trp63α</sub> (1761 bp), ΔN<sub>Trp63β</sub> (1382 bp), ΔN<sub>Trp63γ</sub> (1125 bp), and Actb (β-actin control) (244 bp) in mammary carcinomas classified as non-basal (1–5) or basal (6–10) carcinomas by cDNA array analysis. Size of DNA ladder bands indicated in M (marker) lane. (B) Representative images of histology (H&E) and expression patterns of TRP63 and ΔNTRP63 in the same non-basal (1–5) and basal (6–10) carcinomas analyzed in (A). (C) Representative images illustrating expression patterns of cytokeratin 5 (KRT5) and smooth muscle actin (ACTA2) from basal and non-basal carcinomas corresponding to lanes illustrated in (A). H&E: large panels taken at 10× magnification, insets at 40× magnification. Scale bar = 50 μM. TRP63 and ΔNTRP63 panels were taken at 40× magnification. Scale bar = 10 μM. Open arrowheads and closed arrows indicate cells with representative immunohistochemical staining.
Expression of Trp63 isoforms in primary cell lines established from mouse mammary cancers with mutated Brca1 and Trp52 genes. (A) Normalized read coverage across the Trp63 locus viewed through the integrative genomics viewer illustrates the relative expression levels of different exons of the Trp63 gene in primary cancer cells from mice with two intact Brca1 alleles (Brca1+/–, Trp52+/–; Brca1+/+ /MMTV-Cre/Trp52+–/–; Tet-Op-CYP19A1/MMTV–rtTA mice), one intact Brca1 allele (Brca1+/–, Trp52+/–; Brca1+/+ /MMTV-Cre/Trp52+–/–; Tet-Op-CYP19A1/MMTV–rtTA mice), and two disrupted Brca1 alleles (Brca1–/–, Trp52+/–; Brca1+/+ /MMTV-Cre/Trp52–/–; Tet-Op-CYP19A1/MMTV–rtTA mice) cultured under conditions maintaining epithelial cell differentiation (E) or permissive for epithelial mesenchymal transition (EMT). Note that exons demonstrating expression are all contained within ΔNTrp63 spliced forms. (B) Bar graphs illustrating relative fragments per kilobase of transcript per million mapped (FPKM) Trp63 reads in the primary cancer cells with varying numbers of Brca1 alleles (A) cultured under conditions favoring epithelial cell differentiation (dark gray) or permissive for EMT (light gray).

Discussion

These studies, for the first time, provide insight into the chromatin landscape of the Trp63 locus in mouse mammary epithelium and provide genetic evidence that STAT5 is a negative regulator of this gene. Two classes of Trp63 isoforms had been reported, the long TA and short ΔN forms, which are derived from transcripts originating from the distal and proximal promoters respectively. Based on ChIP-seq data, H3K4me2 and H3K4me3 activating chromatin marks are associated almost exclusively with the proximal promoter encoding the ΔNTrp63 spliced forms portending the significantly higher levels of ΔNTrp63 as compared with TATrp63 documented by RNA-seq and RT-PCR across normal development and many of the cancers. ΔNTrp63 isoforms are shown to act as survival factors in normal and cancer cells of epithelial origin (Mills et al. 1999, Lee et al. 2006, Rocco et al. 2006, Dugani et al. 2009) compatible with a role for ΔNTrp63 in promoting cancer cell survival in cancers that express it. However, it is clear both in human breast cancers (Laakso et al. 2005, Jumppanen et al. 2007, Moriya et al. 2009, Thike et al. 2010b, Lehmann et al. 2011, Shao et al. 2011, Furuya et al. 2012, Yao & Chen 2012, Masuda et al. 2013, Shekhar et al. 2013) and in mammary cancers that develop in Brca1f11/f11/MMTV-Cre/Trp53+–/– mice (shown herein, Nakles et al. 2013) that not all cancers express ΔNTrp63, indicating that it is not absolutely required for cancer development.

Breast cancer is a heterogeneous disease and the Brca1f11/f11/MMTV-Cre/Trp53+–/– mice develop a spectrum of triple-negative breast cancer subtypes (Herschkowitz et al. 2007). Percentages of cancers showing nuclear-localized TRP63 reported herein parallel previously reported results with ~50% of the cancers showing strong protein expression on IHC, which is associated with concomitant KRT15 expression (Nakles et al. 2013) but is more variably accompanied by ACTA2 expression. This expression pattern is different from normal mature myoepithelial cells, where all three of these markers are expressed synchronously, but parallels results from human breast cancers, where discordant expression is also found (Laakso et al. 2005, Jumppanen et al. 2007). It has been recently reported that TRP63 detected in cancers represents ΔNTrp63 isoform expression predominantly, confirming that the Brca1f11/f11/MMTV-Cre/Trp53+–/– mouse model has the same pattern of TRP63 and ΔNTrp63 isoform expression described in human breast cancers (Su et al. 2013). This is the form most closely linked to MEC survival (Yallowitz et al. 2014), compatible with it mediating a role in cancer development.
cell survival in this model. TRP63 also has been linked to maintenance of PI-MECs that in turn can have an impact on the extent of pregnancy-induced ErbB2 cancer development (Yallowitz et al. 2014). Herein, cancers were studied from nulliparous Brca1\textsuperscript{F11/l1/MMTV-Cre/Trp53+/−} mice but it remains possible that TRP63 contributed to survival of cancer progenitor cells. The RNA studies provide novel information on expression of spliced forms of Trp63 in cancers, illustrate that they are not all identical in cancers (even developing within one genetically engineered mouse model), and establish the higher sensitivity of RNA for detection of gene activity. The spectrum of Trp63 expression in the different histological cancer types developing in this model and the fact that cancers develop in nulliparous mice at high frequency suggest that it would be a suitable preclinical model for the next set of investigations exploring the impact of Trp63 deletion on cancer pathophysiology and therapeutic outcome. Nrg1 is expressed in the primary cell lines developed from the cancers with one and two disrupted Brca1 alleles, providing linked in vivo and in vitro models for study of a possible role for Nrg1 in cancer development linked to loss of Brca1 function. A similar approach to develop cell lines from human breast cancers with mutated Brca1 alleles could be used to develop parallel human models (Liu et al. 2012). In both humans with mutated BRCA1 and mice with disrupted Brca1 alleles, accumulation of altered luminal progenitor cells is suggested to represent the population from which eventual cancer cells are derived (Lim et al. 2009, Smart et al. 2011).

Here, STAT5 emerged as a negative regulator of Trp63 with genetic reduction of STAT5 levels resulting in increased Trp63 levels, a new finding. This negative regulation is consistent with the cellular localization of TRP63 to basal myoepithelial cells, while STAT5 is primarily expressed in mammary luminal cells. This expands upon the interplay between TRP63 and activated STAT5 in mammary epithelium mediated by Nrg1 (Forster et al. 2014). TRP63 expression in mammary myoepithelial cells is required for activation of STAT5 in luminal cells, but then STAT5 might act to repress Trp63 expression in these luminal cells. Similar to other studies, these results demonstrate TRP63 expression throughout all post-pubertal mammary gland development phases with ΔNTRP63 expression predominating (Parsa et al. 1999, Forster et al. 2014, Yallowitz et al. 2014) but extend them by documenting the expression patterns of all six different isoforms and showing that loss of Brca1 does not alter the basic pattern of nuclear localization in myoepithelial cells. PGR binding was also localized to ΔNTrp63 promoter region and interactions between STAT5 and PGR have been reported. PGR is also expressed primarily in luminal mammary cells and may also be a negative regulator of ΔNTrp63, but this hypothesis needs to be directly tested in vivo. Experiments confirmed that BRCA1 is not essential for Trp63 expression but, in agreement with the previous literature, demonstrated that increased Trp63 expression was associated with higher levels of full-length Brca1. Expression of TRP63 and ΔNTRP63 in basal myoepithelial cells first appears during normal mammary gland pubertal differentiation. This study demonstrated that expression of Trp63 is also linked positively to differentiation in mammary cancers, both in vivo and in vitro. Significantly, prevalence of TRP63 in cancers developing in Brca1\textsuperscript{F11/l1/MMTV-Cre/Trp53+/−} mice is increased from 50 to 92% by treatment with efatutazone, a differentiating agent that is a ligand for peroxisome proliferator-activated receptor gamma (PPARγ), and demonstrated a lower prevalence of TRP63 expression in spindloid cancers as compared with more differentiated cancer subtypes (Nakles et al. 2013). Herein, we showed that transcriptional regulatory mechanisms activated during EMT (Lindsay et al. 2011) include Trp63 down-regulation.

It is challenging to assign RNA expression patterns to a specific cell type when working with whole tissue. Experiments herein demonstrated that TRP63 and ΔNTRP63 remained nuclear-localized to basal myoepithelial cells across different stages of reproductive development. RNA expression levels can be measured by a variety of methods. Herein, we showed that the RT-PCR technology used was more sensitive than the conditions employed for RNA-seq (40–100 million reads) for detection of the TATTrp63 isoforms. The lower levels of Trp63 expression found herein during lactation as compared with nulliparous mice may be secondary to the lower percentage of myoepithelial cells as compared with luminal cells at that time point, as expression of Trp63 in isolated luminal cells is very low and not significantly changed in isolated basal cells between those two time points (Forster et al. 2014). Expression does consistently appear to be increased with differentiation when changes in cell population types is not a factor as observed with the increase during the estrous cycle and with differentiation in cell culture as shown herein.

In conclusion, this study contributes new information on the regulation of Trp63, implicating STAT5 and defining the role of BRCA1 and demonstrating the impact of differentiation. RNA isoform and protein expression patterns across normal mammary gland development,
including reproductive cycles, were explicitly written, illustrating a predominant role for the ΔNTRP63 isoforms.

A validated genetically engineered mouse model suitable for further studies investigating the impact of TRP63 on mammary disease and therapy was presented.

Declaration of interest
S Assefnia, K Kang, S Groeneveld, D Yamaji, S Dabydeen, A Alamri, L Hennighausen and P A Furth declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported. Georgetown University has submitted a patent application for the cell reprogramming technology described, on which X Liu is an inventor. The intellectual property is under an exclusive license to Propagenix in which Georgetown University has a founding equity interest.

Acknowledgements
We thank M Carla Cabrera and Sarah L Millman for acquisition of immunohistochemistry images (Fig. 4). We thank M Carla Cabrera and Sarah L Millman for acquisition of libraries.

Author contribution statement
S Assefnia and K Kang contributed equally to this work, and with L Hennighausen and P A Furth designed, performed, and analyzed experiments, prepared figures, and wrote the manuscript. S Groeneveld, A Alamri, and X Liu cultured primary cells. S Dabydeen prepared RNA-seq libraries.

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