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

Shahin Assefnia[1,*], Keunsoo Kang[2,3,*], Svenja Groeneveld[1,4], Daisuke Yamaji[2], Sarah Dabydeen[1], Ahmad Alamri[1,5], Xuefeng Liu[6], Lothar Hennighausen[2] and Priscilla A Furth[1,7]

[1]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University, 3970 Reservoir Road NW, Research Building, Room 520A, Washington, District of Columbia 20057, USA
[2]Laboratory of Genetics and Physiology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 8 Center Drive, Bethesda, Maryland 20892-0822, USA
[3]Department of Microbiology, Dankook University, Cheonan 330-714, Republic of Korea
[4]Department Pharmazie, Ludwig-Maximilians-Universität München, München, Germany
[5]College of Medical Sciences, King Khalid University, Abha, Saudi Arabia
Departments of [6]Pathology and [7]Medicine, Lombardi Comprehensive Cancer Center, Georgetown University, 3970 Reservoir Road NW, Research Building, Room 520A, Washington, District of Columbia 20057 USA
[*](S Assefnia and K Kang contributed equally to this work)

Correspondence should be addressed to P A Furth
Email paf3@georgetown.edu

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.

Key Words
- mammary gland
- gene regulation
- molecular genetics
- oncogene
- neoplasia

Endocrine-Related Cancer (2014) **21**, 443–457

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

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 (ΔN) isoform. Both TA and ΔN 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 TRP53 (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). TA*Trp63* isoforms are expressed before ΔN*Trp63* 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 appears to be essential for maintaining the proliferative and regenerative abilities of the stem cell pool in stratified epithelial structures (Pellegrini *et al*. 2001, Senoo *et al*. 2007, Pignon *et al*. 2013) and glandular mammary tissue (Yallowitz *et al*. 2014). TRP63 has been reported to regulate cell survival (Liefer *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 ΔN 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 (Montagner *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 parity-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 progesterone 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, Yamaji *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).

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

The presence of TRP63 is a proposed biomarker for basal cancer (Thike *et al*. 2010*a*, 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 ΔN*Trp63* 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* (Herschkowitz *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, $Brca1^{floxed\ exon\ 11\ (f11)/f11/mouse\ mammary\ tumor\ virus\ (MMTV)\text{-}Cre/Trp53+/+}$, $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$, $Brca1^{f11/WT/MMTV\text{-}Cre/Trp53+/-}$, and $Brca1^{WT/WT/MMTV\text{-}Cre/Trp53+/-/tet\text{-}op\text{–}CYP19A1/MMTV\text{–}rtTA}$ mice were identified by PCR (Jones *et al*. 2005; Transnetyx, 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 CO_2 narcosis and tissues were removed at necropsy following the guidelines approved by the GUACUC/NIHIACUC. 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 $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$, $Brca1^{f11/WT/MMTV\text{-}Cre/Trp53+/-}$, and $Brca1^{WT/WT/MMTV\text{-}Cre/Trp53+/-/tet\text{-}op\text{–}CYP19A1/MMTV\text{–}rtTA}$ 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 (Neomarkers, Fremont, CA, USA); TATRP63, sc-8608, 1:40; ΔNTRP63, sc-8609, 1:40; ESR1, sc-542, 1:750; PGR, sc-538, 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, PRB-160P, 1:1000 (Covance Lab, Princeton, NJ, USA); and ACTA2, 1184-1, 1:500 (Epitomics, Burlingame, CA, USA). Cancers were classified as triple-negative by gene expression array analyses (Herschkowitz *et al*. 2007) and/or by absence of ESR1, PGR, and HER2 $\leq 2+$ on IHC. IHC specificity was tested by omission of the primary

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

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% CO_2 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* (forward (F), CCCAGAGGTCTTCCAGCATATCT and reverse (R), TCAACTCGATGGGCTGTACTG); SApSh3D-SpC for *ΔNTrp63* (F, CCTGGAAAACAATGCCCAGACT and R, AGGAGCCCCAGGTTCGT); SApSh3A-ESCR for *Trp63α* (F, GGGCTGACCACCATCTATCA and R, GTCGGA-ACTGTTCAGGGATCTT); and SApSh3G-EDDp for *Trp63γ* (F, CAGCACCAGCACCTACTTCA and R, GCTCCACAAG-CTCATTCCTGAA) with expression levels normalized to 18S (Hs99999901_s1). Means and S.E.M.s were analyzed using the Mann–Whitney *U* test (GraphPad Software, San Diego, CA, USA). The *P* values of <0.05 were considered statistically significant. *Trp63*, TA*Trp63*, ΔN*Trp63*, *Trp63α*, *Trp63*β, and *Trp63γ* RT-PCR was performed using published primers and conditions (Kurita *et al.* 2005) with *Actb* as control. For RNA-seq, RNA was isolated from frozen tissue or cell pellets (TRIzol, Invitrogen), purified (RNeasy Plus Mini Kit, Qiagen), and analyzed (Nanodrop, Thermo-Scientific (Wilmington, DE, USA) and Agilent Bioanalyzer 2100, Agilent Technologies, Inc. (Santa Clara, CA, USA)). Then RNA was converted to cDNA (SuperScript II, Invitrogen), sequencing libraries were prepared (TruSeq RNA Sample Preparation Kit, Illumina, San Diego, CA, USA) and analyzed (Nanodrop, Agilent Bioanalyzer 2100), and paired-end sequencing was performed (HiSeq 2000, Illumina). Read quality was determined using FastQC (http://www.bioinformatics.babraham.ac.uk/project/fastqc), and contaminated adaptor portions were trimmed using Trim Galore (http://www.bioinformatics.babraham.ac.uk/project/trim_galore). Reads were aligned to mouse reference genome mm9 assembly, TopHat (Kim *et al.* 2013), and abundance of transcripts (fragments per kilobase of transcript per million mapped (FPKM) reads) was estimated. The mapped reads were analyzed using Cuffdiff (Trapnell *et al.* 2010) and visualized using the integrative genomics viewer (Robinson *et al.* 2011). GEO (http://www.ncbi.nlm.nih.gov/geo/) obtained ChIP-seq data (STAT5 and trimethylated lysine 4 histone H3 (H3K4me3) (GSE40930 and GSE31578) (Yamaji *et al.* 2013, Zhang *et al.* 2012); PGR (GSE42887) (Lain *et al.* 2013); dimethylated lysine 4 histone H3 (H3K4me2) (GSE25105) (Rijnkels *et al.* 2013); and RNA-seq (GSE37646)). ChIP-seq data were reanalyzed using the HOMER Software (http://homer.salk.edu/homer/) (Heinz *et al.* 2010, Kang *et al.* 2014).

Results

Histone modifications and transcription factor binding at the *Trp63* locus

Regulatory features of the *Trp63* gene in mouse mammary tissue were analyzed by scanning the locus for pertinent histone modifications and binding of transcription factors STAT5 and PGR, known to control gene expression in mammary epithelium, through mining ChIP- and RNA-seq data from WT mammary gland (Zhang *et al.* 2012, Lain *et al.* 2013, Rijnkels *et al.* 2013, Yamaji *et al.* 2013, Kang *et al.* 2014; Fig. 1). H3K4me3 marks are indicative of active or poised promoters and H3K4me2 marks have been associated with enhancers. Definitive H3K4me3 and H3K4me2 marks were observed only over the proximal promoter that yields ΔN*Trp63* transcripts (Fig. 1A). H3K4me2 marks coincided with STAT5 binding at the proximal but not distal promoter. Binding of STAT5 at the same site in liver tissue supports the importance of this

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

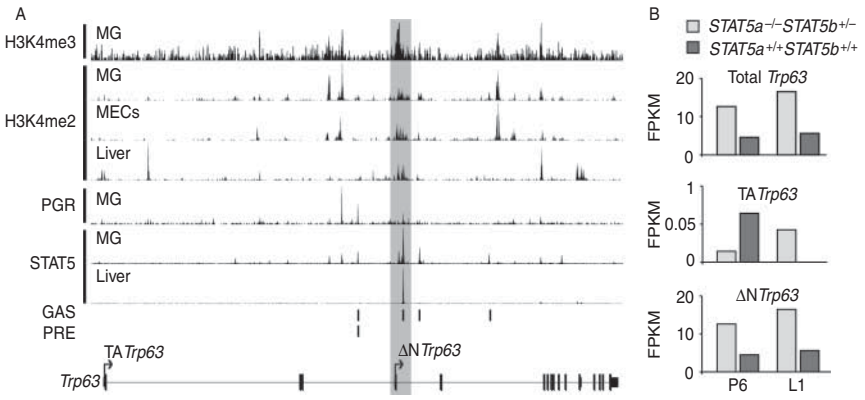

Figure 1

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 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. (B) Bar graphs illustrating relative fragments per kilobase of transcript per million mapped (FPKM) reads of *Trp63*, TA*Trp63*, and ΔN*Trp63* 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).

site. 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 ΔN*Trp63* isoforms were much higher than those of TA*Trp63* 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 ΔN*Trp63* 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 ΔN*Trp63*, total *Trp63*, TA*Trp63*, *Trp63α*, and

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

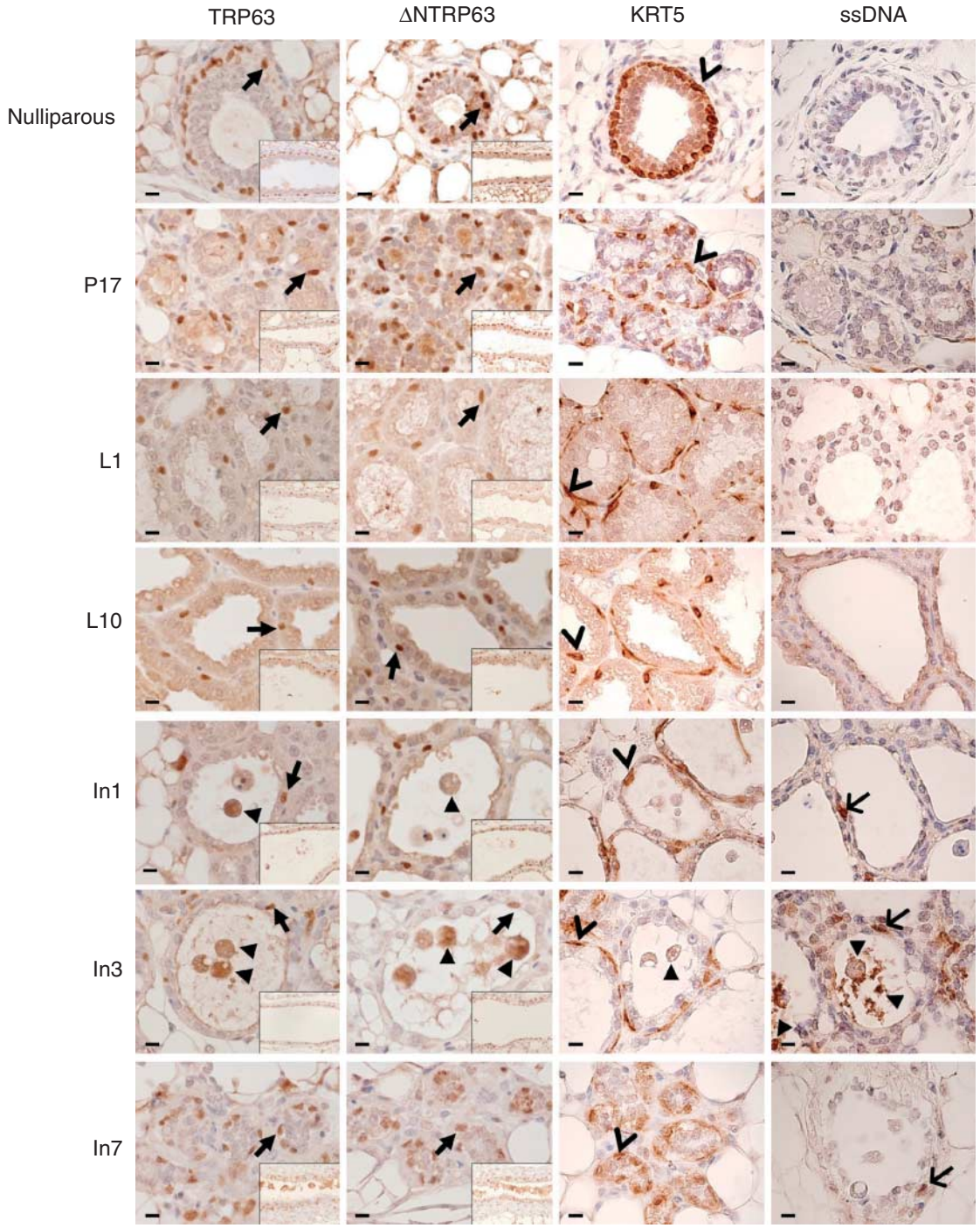

Figure 2

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 arrowhead points to representative cells shed into the lumen demonstrating KRT5 expression. ssDNA: arrows 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.

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

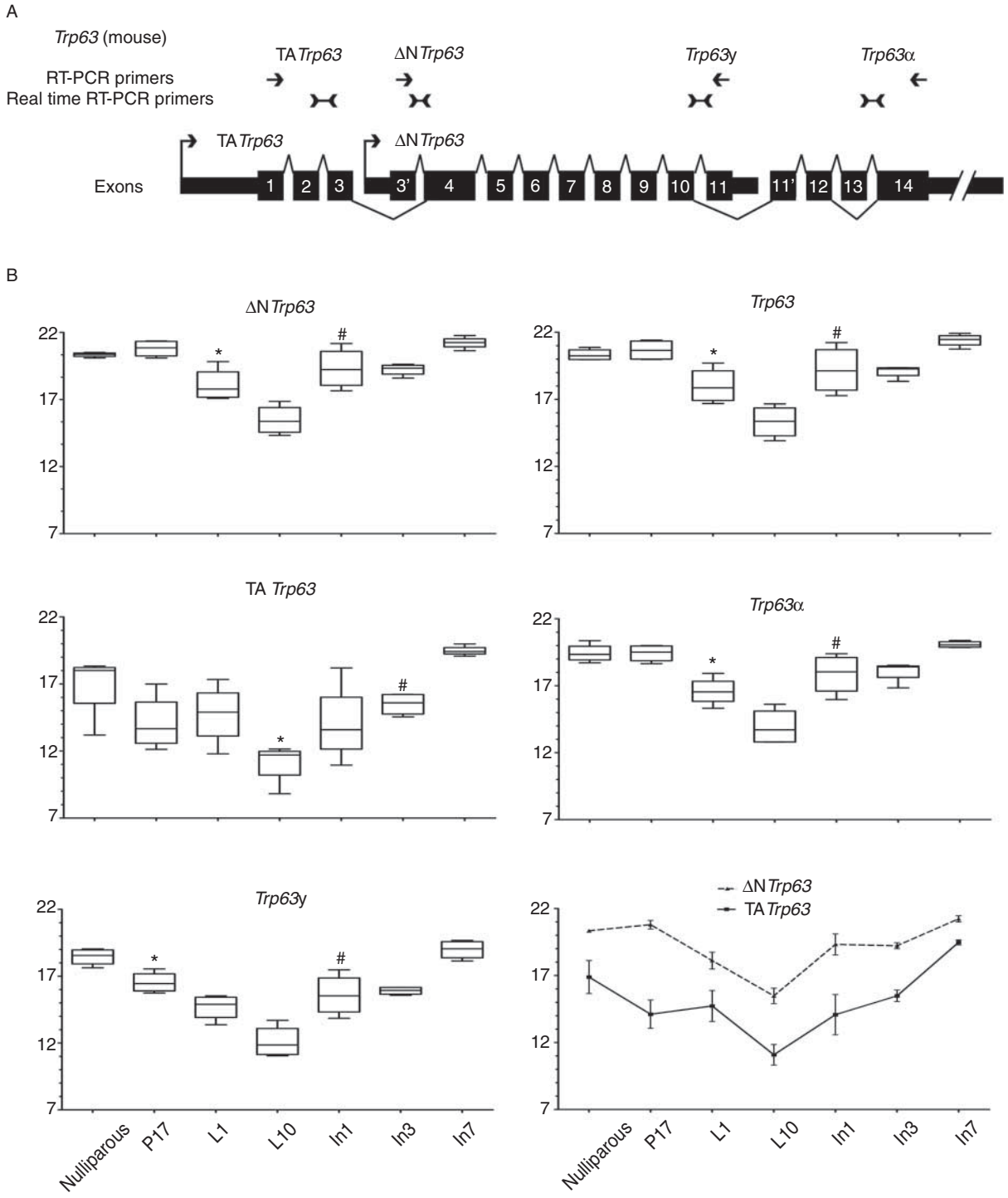

Figure 3

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 ΔN*Trp63*, 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 ΔN*Trp63* 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).

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

*Trp63*γ 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 TA*Trp63* and ΔN*Trp63* promoters, and 3′-termini of the *Trp63*α and *Trp63*γ spliced forms (Fig. 3A). It was not possible to design primers to specifically detect the *Trp63*β 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 ΔN*Trp63* and TA*Trp63* spliced forms (Fig. 3B). Expression levels of both spliced forms were lowest at L10 but the kinetics of change differed. ΔN*Trp63* expression was significantly lower at L1 ($P<0.05$; Mann–Whitney *U* test compared with nulliparous, P17) while expression levels of TA*Trp63* 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 ΔN*Trp63* compared with TA*Trp63*, the significant drop in total *Trp63* occurred at L1 ($P<0.05$; Mann–Whitney *U* test compared with nulliparous, P17) as shown specifically for ΔN*Trp63*. *Trp63*α forms also dropped significantly at L1 ($P<0.05$; Mann–Whitney *U* test compared with nulliparous, P17) while *Trp63*γ forms showed a different pattern dropping significantly at P17 ($P<0.05$; Mann–Whitney *U* test compared with nulliparous). Expression of ΔN*Trp63*, total *Trp63*, *Trp63*α, and *Trp63*γ all rose significantly at In1 (all $P<0.05$; Mann–Whitney *U* test compared with L1) but again the pattern of TA*Trp63* was unique, rising significantly only at In3. Line graphs indicated that expression of ΔN*Trp63* was higher than that of TA*Trp63* 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 ($Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/+}$ 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 $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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, ΔN*Trp63* spliced forms were expressed at higher levels than TA*Trp63* 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

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

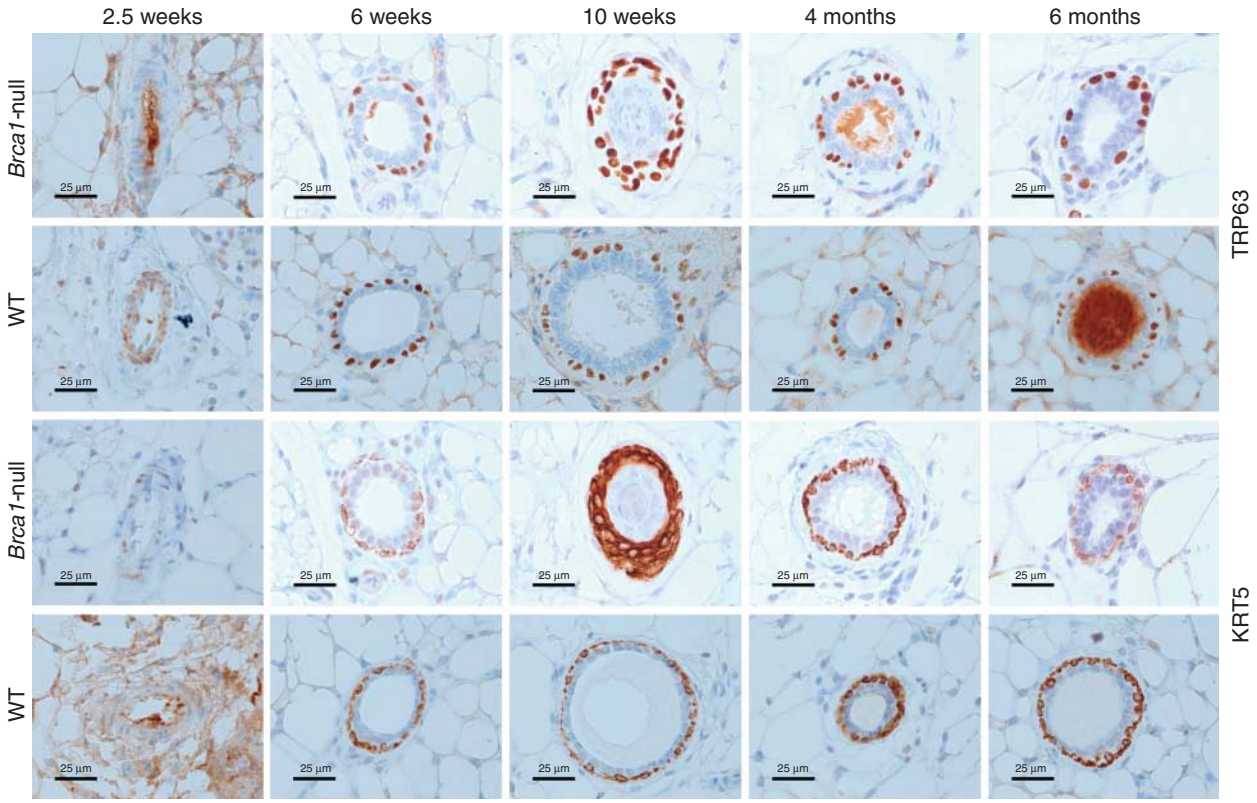

Figure 4

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 ($Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/+}$) 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.

spindloid cancers showed a different pattern with undetectable RNA expression in one correlated with cytoplasmic staining (2) and relatively equal TA and ΔN*Trp63*γ 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. $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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*. 2010*b*). 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 ΔN*Trp63* 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 $Brca1^{WT/WT/MMTV\text{-}Cre/Trp53+/-/tet\text{-}op\text{-}CYP19A1/MMTV\text{-}rtTA}$ mice, one intact *Brca1* allele ($Brca1^{+/-}Trp53^{+/-}$) from $Brca1^{f11/WT/MMTV\text{-}Cre/Trp53+/-}$ mice, and two disrupted *Brca1* alleles ($Brca1^{-/-}Trp53^{+/-}$) from $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

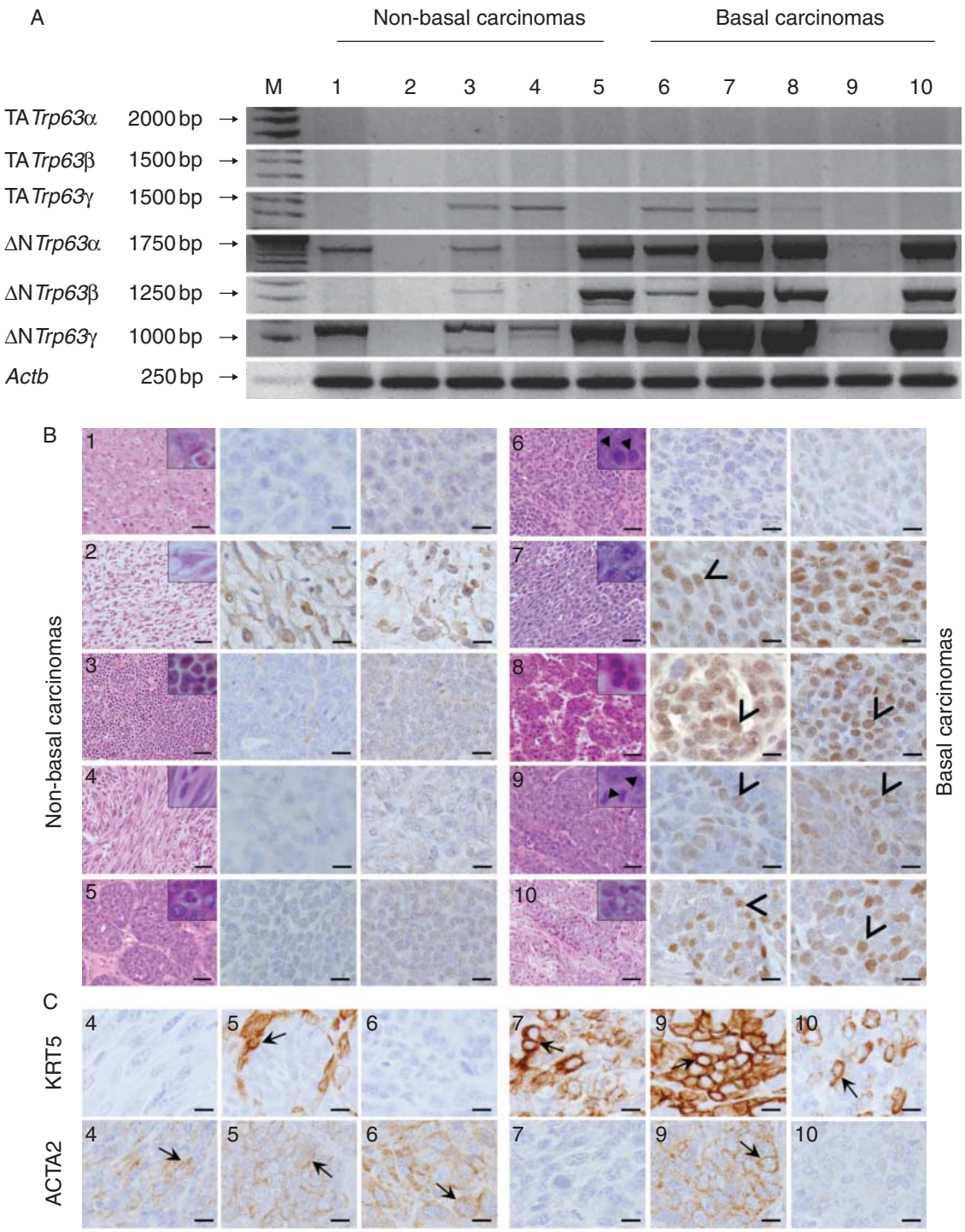

Figure 5

Expression levels of different *Trp63* isoforms and basal markers in mammary carcinomas developing in Brca1$^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ mice classified as either non-basal or basal. (A) Relative expression levels of TA*Trp63*α (2043 bp), TA*Trp63*β (1664 bp), TA*Trp63*γ (1407 bp), ΔN*Trp63*α (1761 bp), ΔN*Trp63*β (1382 bp), ΔN*Trp63*γ (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.

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

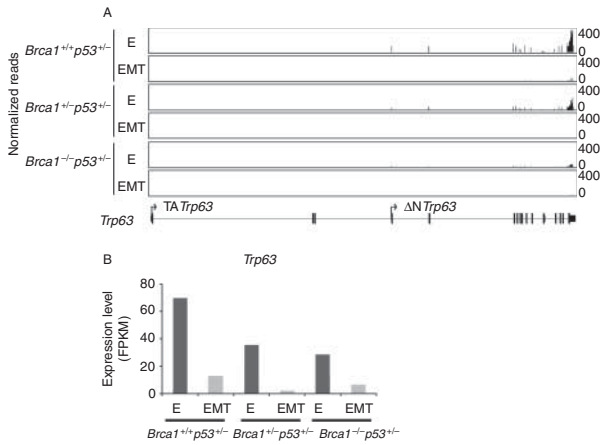

Figure 6

Expression of *Trp63* isoforms in primary cell lines established from mouse mammary cancers with mutated *Brca1* and *Trp53* 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 ($Brca1^{+/+}Trp53^{+/-}$; $Brca1^{WT/WT/MMTV\text{-}Cre/Trp53+/-/tet\text{-}op\text{-}CYP19A1/MMTV\text{-}rtTA}$ mice), one intact *Brca1* allele ($Brca1^{+/-}Trp53^{+/-}$; $Brca1^{f11/WT/MMTV\text{-}Cre/Trp53+/-}$ mice), and two disrupted *Brca1* alleles ($Brca^{-/-}Trp53^{+/-}$; $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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 ΔN*Trp63* 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).

documented in E cells when compared with EMT cells, while significantly higher fold expression of genes linked to EMT (*Vim*, greater than threefold; *Snail1*, greater than eightfold; *Twist1*, greater than fourfold; and *Twist2*, greater than sevenfold) was found in EMT cells when compared with E cells (Cuffdiff, $P<0.05$). The ΔN*Trp63* genes were the dominant isoforms expressed across genotypes and culture conditions (Fig. 6A). Disruption of *Brca1* reduced *Trp63* expression levels by approximately twofold in both E and EMT cells (Fig. 6B). Significantly, *Trp63* expression levels were at least fourfold higher in E cells of all genotypes (Fig. 6B). These *in vitro* results paralleled the *in vivo* expression differences in the adenocarcinomas as compared with spindloid cancers and indicate that *Trp63* is intrinsically down-regulated during the process of EMT. Because loss of *Trp63* has been linked to lower expression levels of *Nrg1* (Forster *et al.* 2014, Yallowitz *et al.* 2014), expression levels were compared in the different cell lines. Expression levels were similar in E and EMT cells with one disrupted *Brca1* allele (9 and 10 FPKM respectively), but reduced by greater than threefold in EMT (4 FPKM) as compared with E (13 FPKM) cells with two disrupted *Brca1* alleles (Cuffdiff; $P<0.05$).

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 ΔN*Trp63* spliced forms portending the significantly higher levels of ΔN*Trp63* as compared with TA*Trp63* 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.* 2010*b*, 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 $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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 $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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 KRT5 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 $Brca1^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

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*$^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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 Δ*Trp63* 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 Δ*Trp63*, 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*$^{f11/f11/MMTV\text{-}Cre/Trp53+/-}$ 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 TA*Trp63* 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,

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-14-0032

Published by Bioscientifica Ltd.

including reproductive cycles, were explicitly defined, 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.

Funding

This research was supported in part by NIH NCI RO1 CA112176 (P A Furth), DOD W81XWH-07-1-058 (P A Furth), NIH IG20RR025828 (Rodent Barrier Facility Equipment), NIH 1R21CA180524 (X Liu), Cherry Blossom Breast Cancer Foundation Grant (X Liu), and NIH NCI 5P30CA051008 (Histology and Tissue, Genomics and Epigenomics, and Animal Shared Resources). Part of this research was funded by the Intramural Research Program of NIDDK/NIH.

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.

Acknowledgements

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

References

Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JF, Nicholson RI & Ellis IO 2004 Expression of luminal and basal cytokeratins in human breast carcinoma. *Journal of Pathology* **203** 661–671. (doi:10.1002/path.1559)

Bankfalvi A, Ludwig A, De-Hesselle B, Buerger H, Buchwalow IB & Boecker W 2004 Different proliferative activity of the glandular and myoepithelial lineages in benign proliferative and early malignant breast diseases. *Modern Pathology* **17** 1051–1061. (doi:10.1038/modpathol.3800082)

Barton CE, Johnson KN, Mays DM, Boehnke K, Shyr Y, Boukamp P & Pietenpol JA 2010 Novel p63 target genes involved in paracrine signaling and keratinocyte differentiation. *Cell Death & Disease* **1** e74. (doi:10.1038/cddis.2010.49)

Buckley NE & Mullan PB 2012 BRCA1 – conductor of the breast stem cell orchestra: the role of BRCA1 in mammary gland development and identification of cell of origin of BRCA1 mutant breast cancer. *Stem Cell Reviews* **8** 982–983. (doi:10.1007/s12015-012-9354-y)

Buckley NE, Conlon SJ, Jirstrom K, Kay EW, Crawford NT, O'Grady A, Sheehan K, Mc Dade SS, Wang CW, McCance DJ *et al.* 2011 The ΔNp63 proteins are key allies of BRCA1 in the prevention of basal-like breast cancer. *Cancer Research* **71** 1933–1944. (doi:10.1158/0008-5472.CAN-10-2717)

Celardo I, Grespi F, Antonov A, Bernassola F, Garabadgiu AV, Melino G & Amelio I 2013 Caspase-1 is a novel target of p63 in tumor suppression. *Cell Death & Disease* **4** e645. (doi:10.1038/cddis.2013.175)

Cheung KJ, Gabrielson E, Werb Z & Ewald AJ 2013 Collective invasion in breast cancer requires a conserved basal epithelial program. *Cell* **155** 1639–1651. (doi:10.1016/j.cell.2013.11.029)

Chu WK, Dai PM, Li HL & Chen JK 2008 Transcriptional activity of the ΔNp63 promoter is regulated by STAT3. *Journal of Biological Chemistry* **283** 7328–7337. (doi:10.1074/jbc.M800183200)

Cui Y, Riedlinger G, Miyoshi K, Tang W, Li C, Deng CX, Robinson GW & Hennighausen L 2004 Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Molecular and Cellular Biology* **24** 8037–8047. (doi:10.1128/MCB.24.18.8037-8047.2004)

Dohn M, Zhang S & Chen X 2001 p63α and ΔNp63α can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. *Oncogene* **20** 3193–3205. (doi:10.1038/sj.onc.1204427)

Dugani CB, Paquin A, Fujitani M, Kaplan DR & Miller FD 2009 p63 antagonizes p53 to promote the survival of embryonic neural precursor cells. *Journal of Neuroscience* **29** 6710–6721. (doi:10.1523/JNEUROSCI.5878-08.2009)

Fata JE, Chaudhary V & Khokha R 2001 Cellular turnover in the mammary gland is correlated with systemic levels of progesterone and not 17β-estradiol during the estrous cycle. *Biology of Reproduction* **65** 680–688. (doi:10.1095/biolreprod65.3.680)

Forster N, Saladi SV, van Bragt M, Sfondouris ME, Jones FE, Li Z & Ellisen LW 2014 Basal cell signaling by p63 controls luminal progenitor function and lactation via NRG1. *Developmental Cell* **28** 147–160. (doi:10.1016/j.devcel.2013.11.019)

Frankfurt OS 2004 Immunoassay for single-stranded DNA in apoptotic cells. *Methods in Molecular Biology* **282** 85–101.

Furuya C, Kawano H, Yamanouchi T, Oga A, Ueda J & Takahashi M 2012 Combined evaluation of CK5/6, ER, p63, and MUC3 for distinguishing breast intraductal papilloma from ductal carcinoma *in situ*. *Pathology International* **62** 381–390. (doi:10.1111/j.1440-1827.2012.02811.x)

Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, Cheng JX, Murre C, Singh H & Glass CK 2010 Simple combinations of lineage-determining transcription factors prime *cis*-regulatory elements required for macrophage and B cell identities. *Molecular Cell* **38** 576–589. (doi:10.1016/j.molcel.2010.05.004)

Herschkowitz JI, Simin K, Weigman VJ, Mikaelian I, Usary J, Hu Z, Rasmussen KE, Jones LP, Assefnia S, Chandrasekharan S *et al.* 2007 Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biology* **8** R76. (doi:10.1186/gb-2007-8-5-r76)

Jones LP, Li M, Halama ED, Ma Y, Lubet R, Grubbs CJ, Deng CX, Rosen EM & Furth PA 2005 Promotion of mammary cancer development by tamoxifen in a mouse model of Brca1-mutation-related breast cancer. *Oncogene* **24** 3554–3562. (doi:10.1038/sj.onc.1208426)

Jumppanen M, Gruvberger-Saal S, Kauraniemi P, Tanner M, Bendahl PO, Lundin M, Krogh M, Kataja P, Borg A, Ferno M *et al.* 2007 Basal-like phenotype is not associated with patient survival in estrogen-receptor-negative breast cancers. *Breast Cancer Research* **9** R16. (doi:10.1186/bcr1649)

Kang K, Yamaji D, Yoo KH, Robinson GW & Hennighausen L 2014 Mammary-specific gene activation is defined by progressive recruitment of STAT5 during pregnancy and the establishment of H3K4me3 marks. *Molecular and Cellular Biology* **34** 464–473. (doi:10.1128/MCB.00988-13)

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R & Salzberg SL 2013 TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biology* **14** R36. (doi:10.1186/gb-2013-14-4-r36)

Published by Bioscientifica Ltd.

Koster MI, Dai D, Marinari B, Sano Y, Costanzo A, Karin M & Roop DR 2007 p63 induces key target genes required for epidermal morphogenesis. *PNAS* **104** 3255–3260. (doi:10.1073/pnas.0611376104)

Kurita T, Cunha GR, Robboy SJ, Mills AA & Medina RT 2005 Differential expression of p63 isoforms in female reproductive organs. *Mechanisms of Development* **122** 1043–1055. (doi:10.1016/j.mod.2005.04.008)

Laakso M, Loman N, Borg A & Isola J 2005 Cytokeratin 5/14-positive breast cancer: true basal phenotype confined to BRCA1 tumors. *Modern Pathology* **18** 1321–1328. (doi:10.1038/modpathol.3800456)

Lain AR, Creighton CJ & Conneely OM 2013 Research resource: progesterone receptor targetome underlying mammary gland branching morphogenesis. *Molecular Endocrinology* **27** 1743–1761. (doi:10.1210/me.2013-1144)

Lee HO, Lee JH, Choi E, Seol JY, Yun Y & Lee H 2006 A dominant negative form of p63 inhibits apoptosis in a p53-independent manner. *Biochemical and Biophysical Research Communications* **344** 166–172. (doi:10.1016/j.bbrc.2006.03.128)

Lehmann BD, Bauer JA, Chen X, Sanders ME, Chakravarthy AB, Shyr Y & Pietenpol JA 2011 Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *Journal of Clinical Investigation* **121** 2750–2767. (doi:10.1172/JCI45014)

Li M, Hu J, Heermeier K, Hennighausen L & Furth PA 1996 Apoptosis and remodeling of mammary gland tissue during involution proceeds through p53-independent pathways. *Cell Growth & Differentiation* **7** 13–20.

Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, Young WS, Hennighausen L & Furth PA 1997 Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *PNAS* **94** 3425–3430. (doi:10.1073/pnas.94.7.3425)

Liefer KM, Koster MI, Wang XJ, Yang A, McKeon F & Roop DR 2000 Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Research* **60** 4016–4020.

Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A *et al.* 2009 Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. *Nature Medicine* **15** 907–913. (doi:10.1038/nm.2000)

Lindsay J, McDade SS, Pickard A, McCloskey KD & McCance DJ 2011 Role of ΔNp63γ in epithelial to mesenchymal transition. *Journal of Biological Chemistry* **286** 3915–3924. (doi:10.1074/jbc.M110.162511)

Liu X, Robinson GW & Hennighausen L 1996 Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation. *Molecular Endocrinology* **10** 1496–1506.

Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A & Hennighausen L 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes and Development* **11** 179–186. (doi:10.1101/gad.11.2.179)

Liu H, Fan Q, Zhang Z, Li X, Yu H & Meng F 2008 Basal-HER2 phenotype shows poorer survival than basal-like phenotype in hormone receptor-negative invasive breast cancers. *Human Pathology* **39** 167–174. (doi:10.1016/j.humpath.2007.06.012)

Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, Timofeeva OA, Nealon C, Dakic A, Simic V *et al.* 2012 ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *American Journal of Pathology* **180** 599–607. (doi:10.1016/j.ajpath.2011.10.036)

Marquis ST, Rajan JV, Wynshaw-Boris A, Xu J, Yin GY, Abel KJ, Weber BL & Chodosh LA 1995 The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. *Nature Genetics* **11** 17–26. (doi:10.1038/ng0995-17)

Masuda H, Baggerly KA, Wang Y, Zhang Y, Gonzalez-Angulo AM, Meric-Bernstam F, Valero V, Lehmann BD, Pietenpol JA, Hortobagyi GN *et al.* 2013 Differential response to neoadjuvant chemotherapy among 7 triple-negative breast cancer molecular subtypes. *Clinical Cancer Research* **19** 5533–5540. (doi:10.1158/1078-0432.CCR-13-0799)

Mattiske S, Ho K, Noll JE, Neilsen PM, Callen DF & Suetani RJ 2013 TAp63 regulates oncogenic miR-155 to mediate migration and tumour growth. *Oncotarget* **4** 1894–1903.

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR & Bradley A 1999 p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398** 708–713. (doi:10.1038/19531)

Montagner M, Enzo E, Forcato M, Zanconato F, Parenti A, Rampazzo E, Basso G, Leo G, Rosato A, Bicciato S *et al.* 2012 SHARP1 suppresses breast cancer metastasis by promoting degradation of hypoxia-inducible factors. *Nature* **487** 380–384. (doi:10.1038/nature11207)

Moriya T, Kozuka Y, Kanomata N, Tse GM & Tan PH 2009 The role of immunohistochemistry in the differential diagnosis of breast lesions. *Pathology* **41** 68–76. (doi:10.1080/00313020802563544)

Murray-Zmijewski F, Lane DP & Bourdon JC 2006 p53/p63/p73 isoforms: an orchestra of isoforms to harmonise cell differentiation and response to stress. *Cell Death and Differentiation* **13** 962–972. (doi:10.1038/sj.cdd.4401914)

Nakles RE, Kallakury BV & Furth PA 2013 The PPARγ agonist efatutazone increases the spectrum of well-differentiated mammary cancer subtypes initiated by loss of full-length BRCA1 in association with TP53 haploinsufficiency. *American Journal of Pathology* **182** 1976–1985. (doi:10.1016/j.ajpath.2013.02.006)

Nylander K, Vojtesek B, Nenutil R, Lindgren B, Roos G, Zhanxiang W, Sjostrom B, Dahlqvist A & Coates PJ 2002 Differential expression of p63 isoforms in normal tissues and neoplastic cells. *Journal of Pathology* **198** 417–427. (doi:10.1002/path.1231)

Oh JE, Kim RH, Shin KH, Park NH & Kang MK 2011 ΔNp63 protein triggers epithelial–mesenchymal transition and confers stem cell properties in normal human keratinocytes. *Journal of Biological Chemistry* **286** 38757–38767. (doi:10.1074/jbc.M111.244939)

Parsa R, Yang A, McKeon F & Green H 1999 Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *Journal of Investigative Dermatology* **113** 1099–1105. (doi:10.1046/j.1523-1747.1999.00780.x)

Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, Ponzin D, McKeon F & De Luca M 2001 p63 identifies keratinocyte stem cells. *PNAS* **98** 3156–3161. (doi:10.1073/pnas.061032098)

Piccolo S, Enzo E & Montagner M 2013 p63, Sharp1, and HIFs: master regulators of metastasis in triple-negative breast cancer. *Cancer Research* **73** 4978–4981. (doi:10.1158/0008-5472.CAN-13-0962)

Pietsch EC, Sykes SM, McMahon SB & Murphy ME 2008 The p53 family and programmed cell death. *Oncogene* **27** 6507–6521. (doi:10.1038/onc.2008.315)

Pignon JC, Grisanzio C, Geng Y, Song J, Shivdasani RA & Signoretti S 2013 p63-expressing cells are the stem cells of developing prostate, bladder, and colorectal epithelia. *PNAS* **110** 8105–8110. (doi:10.1073/pnas.1221216110)

Ribeiro-Silva A, Ramalho LN, Garcia SB, Brandao DF, Chahud F & Zucoloto S 2005 p63 correlates with both BRCA1 and cytokeratin 5 in invasive breast carcinomas: further evidence for the pathogenesis of the basal phenotype of breast cancer. *Histopathology* **47** 458–466. (doi:10.1111/j.1365-2559.2005.02249.x)

Rijnkels M, Freeman-Zadrowski C, Hernandez J, Potluri V, Wang L, Li W & Lemay DG 2013 Epigenetic modifications unlock the milk protein gene loci during mouse mammary gland development and differentiation. *PLoS ONE* **8** e53270. (doi:10.1371/journal.pone.0053270)

Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G & Mesirov JP 2011 Integrative genomics viewer. *Nature Biotechnology* **29** 24–26. (doi:10.1038/nbt.1754)

Rocco JW, Leong CO, Kuperwasser N, DeYoung MP & Ellisen LW 2006 p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell* **9** 45–56. (doi:10.1016/j.ccr.2005.12.013)

Sarrio D, Rodriguez-Pinilla SM, Hardisson D, Cano A, Moreno-Bueno G & Palacios J 2008 Epithelial–mesenchymal transition in breast cancer

Published by Bioscientifica Ltd.

relates to the basal-like phenotype. *Cancer Research* **68** 989–997. (doi:10.1158/0008-5472.CAN-07-2017)

Sbisa E, Mastropasqua G, Lefkimmiatis K, Caratozzolo MF, D'Erchia AM & Tullo A 2006 Connecting p63 to cellular proliferation: the example of the adenosine deaminase target gene. *Cell Cycle* **5** 205–212. (doi:10.4161/cc.5.2.2361)

Schorr K & Furth PA 2000 Induction of bcl-xL expression in mammary epithelial cells is glucocorticoid-dependent but not signal transducer and activator of transcription 5-dependent. *Cancer Research* **60** 5950–5953.

Schorr K, Li M, Bar-Peled U, Lewis A, Heredia A, Lewis B, Knudson CM, Korsmeyer SJ, Jager R, Weiher H *et al.* 1999 Gain of Bcl-2 is more potent than bax loss in regulating mammary epithelial cell survival *in vivo*. *Cancer Research* **59** 2541–2545.

Senoo M, Manis JP, Alt FW & McKeon F 2004 p63 and p73 are not required for the development and p53-dependent apoptosis of T cells. *Cancer Cell* **6** 85–89. (doi:10.1016/j.ccr.2004.06.005)

Senoo M, Pinto F, Crum CP & McKeon F 2007 p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* **129** 523–536. (doi:10.1016/j.cell.2007.02.045)

Shao MM, Liu J, Vong JS, Niu Y, Germin B, Tang P, Chan AW, Lui PC, Law BK, Tan PH *et al.* 2011 A subset of breast cancer predisposes to brain metastasis. *Medical Molecular Morphology* **44** 15–20. (doi:10.1007/s00795-010-0495-2)

Shekhar MP, Kato I, Nangia-Makker P & Tait L 2013 Comedo-DCIS is a precursor lesion for basal-like breast carcinoma: identification of a novel p63/Her2/neu expressing subgroup. *Oncotarget* **4** 231–241.

Smart CE, Wronski A, French JD, Edwards SL, Asselin-Labat ML, Waddell N, Peters K, Brewster BL, Brooks K, Simpson K *et al.* 2011 Analysis of Brca1-deficient mouse mammary glands reveals reciprocal regulation of Brca1 and c-kit. *Oncogene* **30** 1597–1607. (doi:10.1038/onc.2010.538)

Su X, Chakravarti D & Flores ER 2013 p63 steps into the limelight: crucial roles in the suppression of tumorigenesis and metastasis. *Nature Reviews. Cancer* **13** 136–143. (doi:10.1038/nrc3446)

Thike AA, Cheok PY, Jara-Lazaro AR, Tan B, Tan P & Tan PH 2010*a* Triple-negative breast cancer: clinicopathological characteristics and relationship with basal-like breast cancer. *Modern Pathology* **23** 123–133. (doi:10.1038/modpathol.2009.145)

Thike AA, Iqbal J, Cheok PY, Chong AP, Tse GM, Tan B, Tan P, Wong NS & Tan PH 2010*b* Triple negative breast cancer: outcome correlation with immunohistochemical detection of basal markers. *American Journal of Surgical Pathology* **34** 956–964. (doi:10.1097/PAS.0b013e3181e02f45)

Thike AA, Iqbal J, Cheok PY, Tse GM & Tan PH 2013 Ductal carcinoma *in situ* associated with triple negative invasive breast cancer: evidence for a precursor–product relationship. *Journal of Clinical Pathology* **66** 665–670. (doi:10.1136/jclinpath-2012-201428)

Tran MN, Choi W, Wszolek MF, Navai N, Lee IL, Nitti G, Wen S, Flores ER, Siefker-Radtke A, Czerniak B *et al.* 2013 The p63 protein isoform Np63 inhibits epithelial–mesenchymal transition in human bladder cancer cells: role of MIR-205. *Journal of Biological Chemistry* **288** 3275–3288. (doi:10.1074/jbc.M112.408104)

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ & Pachter L 2010 Transcript assembly and quantification by RNA-seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology* **28** 511–515. (doi:10.1038/nbt.1621)

Truong AB, Kretz M, Ridky TW, Kimmel R & Khavari PA 2006 p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes and Development* **20** 3185–3197. (doi:10.1101/gad.1463206)

Yallowitz AR, Alexandrova EM, Talos F, Xu S, Marchenko ND & Moll UM 2014 p63 is a prosurvival factor in the adult mammary gland during post-lactational involution, affecting PI-MECs and ErbB2 tumorigenesis. *Cell Death and Differentiation* **21** 645–654. (doi:10.1038/cdd.2013.199)

Yamaji D, Na R, Feuermann Y, Pechhold S, Chen W, Robinson GW & Hennighausen L 2009 Development of mammary luminal progenitor cells is controlled by the transcription factor STAT5A. *Genes and Development* **23** 2382–2387. (doi:10.1101/gad.1840109)

Yamaji D, Kang K, Robinson GW & Hennighausen L 2013 Sequential activation of genetic programs in mouse mammary epithelium during pregnancy depends on STAT5A/B concentration. *Nucleic Acids Research* **41** 1622–1636. (doi:10.1093/nar/gks1310)

Yang A & McKeon F 2000 P63 and P73: P53 mimics, menaces and more. *Nature Reviews. Molecular Cell Biology* **1** 199–207. (doi:10.1038/35043127)

Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D & McKeon F 1998 p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Molecular Cell* **2** 305–316. (doi:10.1016/S1097-2765(00)80275-0)

Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C *et al.* 1999 p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398** 714–718. (doi:10.1038/19539)

Yao JY & Chen JK 2012 Roles of p63 in epidermal development and tumorigenesis. *Biomedical Journal* **35** 457–463. (doi:10.4103/2319-4170.104410)

Zhang Y, Laz EV & Waxman DJ 2012 Dynamic, sex-differential STAT5 and BCL6 binding to sex-biased, growth hormone-regulated genes in adult mouse liver. *Molecular and Cellular Biology* **32** 880–896. (doi:10.1128/MCB.06312-11)

Received in final form 14 March 2014
Accepted 31 March 2014
Made available online as an Accepted Preprint
1 April 2014

Published by Bioscientifica Ltd.