ABSTRACT
The identification of multipotent mammary stem cells (MaSCs) has provided an explanation for the unique regenerative capacity of the mammalian gland throughout adult life. However, it remains unclear what genes maintain MaSCs and control their specification into the two epithelial lineages: luminal and basal. LBH is a novel transcription co-factor in the WNT pathway with hitherto unknown physiological function. LBH is expressed during mammary gland development and aberrantly overexpressed in aggressive ‘basal’ subtype breast cancers. Here, we have explored the in vivo role of LBH in mammmopoiesis. We show that in postnatal mammary epithelia, LBH is predominantly expressed in the Lin–CD29<sup>–</sup>CD24<sup>−</sup> population. Upon conditional inactivation of LBH, mice exhibit pronounced delays in mammary tissue expansion during puberty and pregnancy, accompanied by increased luminal differentiation at the expense of basal lineage specification. These defects could be traced to a severe reduction in the frequency and self-renewal/differentiation potential of basal MaSCs. Mechanistically, LBH induces expression of key epithelial stem cell transcription factor Np63α to promote a basal MaSC state and repress luminal differentiation genes, mainly that encoding estrogen receptor α (Erα). Collectively, these studies identify LBH as an essential regulator of basal MaSC expansion/maintenance, raising important implications for its potential role in breast cancer pathogenesis.

KEY WORDS: Limb-bud and heart, Transcription regulation, Mammary gland development, Stem cells, Lineage differentiation, P63, Estrogen receptor, Mouse

INTRODUCTION
Stem cells are vital for adult tissue homeostasis and regeneration, and their deregulation plays a crucial role in human disease, notably cancer (Reya et al., 2001). The mammary gland represents an ideal model system with which to study adult stem cell regulation because of its unique postnatal development (Hennighausen and Robinson, 1998; Watson and Khaled, 2008) and a tremendous regenerative capacity throughout adult life (Visvader and Stingl, 2014). The mammary gland is an epithelial network of ducts and lobules that forms during puberty (4-8 weeks in mice) through elongation and branching of the ducts via rapid expansion of stem/progenitor cells in terminal end buds (TEBs) (Bai and Rohrschneider, 2010; Williams and Daniel, 1983). This structure is composed of an inner layer of luminal cells, expressing luminal keratins (K8+/K18<sup>+</sup>), and an outer layer of basal/myoepithelial cells, expressing basal keratins (K5<sup>+</sup>/K14<sup>+</sup>) (Smith et al., 1990). During pregnancy, the lobulo-alveolar epithelium massively expands in a stem cell-driven manner (Asselin-Labat et al., 2010; Matulka et al., 2007) and luminal alveolar cells differentiate into milk-producing cells to allow lactation, after which the gland regresses to a virgin-like state.

The identification of mammary epithelial cells (MECs) that can reconstitute an entire functional gland at the single cell level upon transplantation into epithelial-free murine mammary fat pads (Kordon and Smith, 1998; Shackleton et al., 2006; Stingl et al., 2006) has suggested that the two mammary epithelial lineages originate from a small population of multipotent mammary stem cells (MaSCs) residing in the basal epithelium and that a differentiation hierarchy exists within these lineages (Visvader and Stingl, 2014). MaSCs with high regenerative capacity are characterized by a CD29<sup>high</sup>/CD49f<sup>high</sup>/CD24<sup>−</sup>/Lin<sup>−</sup>/Sca1−/low surface marker profile, express a basal gene signature and are hormone-receptor negative (Asselin-Labat et al., 2009; Shackleton et al., 2006, 2007; Stingl et al., 2006). Lineage-tracing studies in mice have confirmed the existence of bi-potent basal MaSCs in the mammmary gland in situ (Rios et al., 2014; van Amerongen et al., 2012), as well as identified lineage-restricted unipotent luminal and basal stem/progenitor cells (van Amerongen et al., 2012; Van Keymeulen et al., 2011), which likely together drive postnatal mammary gland morphogenesis in a dynamic fashion (Rios et al., 2014; van Amerongen et al., 2012). Moreover, in vitro and in vivo functional studies suggest that differentiated luminal and myoepithelial cells possess a remarkable plasticity and can dedifferentiate into basal MaSCs (Chafer et al., 2011; Prater et al., 2014). However, the molecular mechanisms governing MaSC regulation in vivo remain ill defined.

LBH (limb-bud and heart) is a highly conserved transcription co-factor in vertebrates, with no homology to known protein families (Al-Ali et al., 2010; Briegel et al., 2005; Briegel and Joyner, 2001). We initially identified Lbh as a novel mouse gene with a unique spatiotemporal expression pattern in the embryonic limb bud and heart (Briegel and Joyner, 2001), whereas others cloned it as a maternal RNA (Xic12) of unknown function in Xenopus that is activated in pluripotent stem cells during early cleavage stages (Paris and Philippe, 1990). Lbh is expressed in additional embryonic and adult tissues, including the gut, brain, peripheral nervous system, and adult tissues, such as the lung, heart, and skin (Paris and Philippe, 1990). It functions with co-factor in the WNT pathway with hitherto unknown physiological function. LBH induces expression of key epithelial stem cell transcription factor Np63α to promote a basal MaSC state and repress luminal differentiation genes, mainly that encoding estrogen receptor α (Erα). Collectively, these studies identify LBH as an essential regulator of basal MaSC expansion/maintenance, raising important implications for its potential role in breast cancer pathogenesis.

system, spleen, lung, kidney and bones (Briegel and Joyner, 2001; Conen et al., 2009; Gawantka et al., 1998; Paris and Philippe, 1990), as well as during specific stages of postnatal mammary gland development (Rieger et al., 2010). Aberrant gain-of-function of LBH is associated with partial trisomy 2p syndrome (Briegel et al., 2005), a human autosomal disorder characterized by congenital heart disease, skeletal growth defects, supernumerary nipples and childhood cancers (Dowa et al., 2006). Overexpression of a Lbh transgene during murine heart development was sufficient to phenocopy the cardiovascular defects observed in these patients (Briegel et al., 2005), whereas retroviral Lbh overexpression in chick embryos delayed bone differentiation (Conen et al., 2009), suggesting LBH is causally implicated in this syndrome. However, the normal physiological function of LBH has remained obscure.

Recently, we showed that Lbh is a direct target gene of the WNT/β-catenin signaling pathway (Rieger et al., 2010), a genetic network fundamental to stem cell control and carcinogenesis in many epithelial tissues (Clevers and Nusse, 2012). WNT also plays a major role in postnatal mammary gland development by promoting the self-renewal and maintenance of basal MaSCs during tissue expansion and homeostasis (Roarty and Rosen, 2010; Zeng and Nusse, 2010). Intriguingly, Lbh mRNA is expressed with a similar pattern to other WNT target genes (Badders et al., 2009; de Visser et al., 2012; Plaks et al., 2013; van Amerongen et al., 2012) in the outer basal epithelial layer and stromal cells at virgin stages, and in the expanding alveolar compartment of pregnant glands, but is virtually absent in terminally differentiated lactating glands (Rieger et al., 2010). Moreover, LBH is aberrantly overexpressed in breast tumors of MMTV-Wnt1 transgenic mice (Rieger et al., 2010), which are enriched in basal MaSCs (Shackleton et al., 2006). Importantly, LBH is abnormally overexpressed in worst prognosis hormone receptor-negative human breast cancers of the ‘basal’ molecular subtype, correlating with WNT pathway hyperactivation (Lamb et al., 2013; Rieger et al., 2010). The strong association between LBH expression and canonical WNT signaling in both normal and cancerous breast tissues prompted us to further explore the role of LBH in mammary epithelial development.

Using a conditional loss-of-function approach in mice, we provide the first in vivo evidence that LBH is required for normal mammosogenesis in the expansion and maintenance of multipotent basal MaSCs. Conversely, LBH represses luminal differentiation, including the expression of estrogen receptor alpha (ERα; Esr1 – Mouse Genome Informatics). Our results further suggest that LBH regulates these processes by acting on the key epithelial stem cell transcription factor ΔNp63.

RESULTS

Differential expression of LBH in distinct mammary epithelial subpopulations

We first determined expression and localization of LBH protein in mammary gland structures by immunohistochemical (IHC) analysis using mammary gland sections from 8-week-old virgin mice. Intense nuclear LBH staining was detected in a subset of cells within the basal epithelial layer and in stromal cells (Fig. 1A), as expected (Rieger et al., 2010). By contrast, LBH was not expressed in luminal cells of the outer epithelial layer of the ducts. Comparative analysis of epithelial lineage marker expression on serial sections showed co-localization of LBH with the basal marker K5 (Krt5 – Mouse Genome Informatics), whereas LBH expression was mutually exclusive with luminal markers, K8 (Krt8 – Mouse Genome Informatics) and ERα (Fig. 1A). Additionally, LBH was expressed in basal cells and individual body cells of TEBs (Fig. 1A).

Furthermore, in adult human breast tissues, LBH was restricted to cells within the baso-myoepithelial layer of the ducts and alveoli (Fig. 1B). Thus, both in murine and human mammary glands, LBH is predominantly expressed in the basal epithelium, whereas it is essentially absent from committed luminal cells.

To identify the epithelial subpopulations expressing LBH, we examined Lbh mRNA expression in FACS-purified luminal Lin−CD29loCD24hi and basal Lin+CD29hiCD24+ MEC populations (referred to as CD29loCD24hi or CD29hiCD24+, respectively) using quantitative RT-PCR (qPCR) (Fig. 1C,D). The successful separation of cell populations was evaluated by luminal (K8) and basal (K5) keratin expression (Fig. 1D). Additionally, Lbh expression was compared with expression of Erα, which primarily marks differentiated luminal cells (Asselin-Labat et al., 2007; Lim et al., 2010), the WNT target gene and basal MaSC marker Axin2 (Jho et al., 2002; Zeng and Nusse, 2010), as well as with expression of the luminal and basal lineage-specific isoforms of epithelial transcription factor p63, TAp63 (Trp63 – Mouse Genome Informatics) and ΔNp63, respectively (Li et al., 2008; Nylander et al., 2002). Notably, Lbh was predominantly expressed in the MaSC-enriched basal K5−Erα−ΔNp63+ fraction, rather than in the luminal K8+Erα+TAp63+ fraction (+6 fold; P<0.01), similar to Axin2 (+3 fold; P<0.05) (Fig. 1D).

Fig. 1. LBH is predominantly expressed in basal mammary epithelial cells. (A) Immunohistochemical staining of serial mammary gland sections from 8-week-old virgin female mice with antibodies specific to LBH and to basal (keratin 5) and luminal (keratin 8, ERα) lineage markers. Left inset: LBH expression in a juvenile terminal end bud (TEB). Scale bars: 25 µm. (B) Immunohistochemical staining of human breast tissues with LBH-specific antibody. Scale bars: 50 µm. (A,B) Right insets show higher magnifications of individual areas; arrows indicate basal cells, arrowheads indicate luminal cells and asterisks indicate stromal cells. (C) FACS segregation of lineage-negative (Lin−: CD31, CD45, TER119−) mammary cells from 8-week-old female mice into luminal (CD29loCD24hi) and basal (CD29hiCD24+) subpopulations. A representative FACS dot plot is shown. (D) qPCR analysis of Lbh and luminal-basal marker expression in FACS-sorted MEC populations relative to Gapdh. Data represent mean±s.e.m. (n≥3 mice); all differences are significant, P<0.05.

To further evaluate whether LBH is associated with WNT-responsive MaSCs, we examined Lhb expression in Lgr5-GFP-CreER T2 reporter mice (Barker et al., 2007). Lgr5 is a direct WNT target gene and a marker for basally located stem cells in different epithelial tissues, including in the mammary gland (de Visser et al., 2012; Plaks et al., 2013). MECs from Lgr5-GFP-CreER T2 mice, which express GFP from the endogenous Lgr5 promoter, were FACs sorted into GFP-positive (GFP +) and GFP-negative (GFP −) cells in combination with a CD24-specific antibody to distinguish between CD24 hi luminal and CD24 lo basal subpopulations (supplementary material Fig. S1A). As predicted, GFP + cells clustered within the CD24 lo fraction, which we confirmed was a basal K5 + population with increased MaSC activity, as determined by qPCR and in vitro mammosphere assays (Dontu et al., 2003), respectively (supplementary material Fig. S1A-C). By contrast, GFP − cells clustered within the CD24 hi K8 + luminal subpopulation. Remarkably, Lhb was highly enriched in the Lgr5-positive GFP +CD24 lo MaSC population to an even greater extent than Axin2, which served as control (+9 fold versus >3 fold; P < 0.01) (supplementary material Fig. S1D). Collectively, these data demonstrate that Lhb is specifically expressed in a rare population of stem-like cells in the basal lineage, raising the notion that LBH may play a role in MaSC biology.

Loss of Lhb impairs postnatal mammary gland development

We next used a loss-of-function strategy to elucidate the in vivo role of LBH in mammosogenesis. We have previously generated mice with a conditional Lhb allele (LhbloxP/loxP), in which exon 2 of Lhb is flanked by two loxP sites, such that Cre-mediated deletion of this exon results in a severely truncated non-functional LBH protein (Lindley and Briegel, 2013). LhbloxP/loxP mice were crossed with transgenic mice expressing Cre under the control of the keratin 14 (K14; Krt14 – Mouse Genome Informatics) promoter, which is active in basal epithelium of the skin and mammary glands (Dassule et al., 2000), and transiently in luminal progenitors at pre-pubertal mammary gland stages (Van Keymeulen et al., 2011). qPCR analysis of FACS-sorted mammary cell populations from K14Cre;LhbloxP/loxP and control K14Cre;LhbloxP/loxP (referred to as wild type) glands showed that no functional Lhb mRNA was expressed in CD29 hiCD24 lo basal cells, its low-level expression in CD29 hiCD24 hi luminal cells was essentially unchanged in mutants compared with the respective cell populations of wild-type mice (Fig. 2A). Immunohistochemistry confirmed the absence of LBH protein in K14Cre;LhbloxP/loxP mammary epithelia, whereas LBH was still expressed in individual stromal cells (Fig. 2A,B).

Prior to puberty (4 weeks of age), K14Cre;LhbloxP/loxP glands were indistinguishable from wild-type glands, suggesting primordial mammosogenesis occurs normally in these mice (supplementary material Fig. S2A). However, in pubescent 6- and 8-week-old mice, when the mammary epithelium normally rapidly expands and invades the fat pad, there was a severe reduction in epithelial outgrowth in Lhb mutant relative to wild-type glands (~60% and ~50%, respectively; P < 0.01) despite the presence of morphologically distinct TEBs (Fig. 2C,D). Ductal extension eventually caught up in Lhb mutant mice at mature virgin stages (11 weeks of age) (supplementary material Fig. S2B). However, parity-induced mammary gland expansion was also perturbed, as evidenced by profoundly reduced alveolar compartments in mid-pregnant and newly lactating K14Cre;LhbloxP/loxP females (supplementary material Fig. S3A). Nevertheless, lactation by Lhb mutant dams proceeded normally (supplementary material Fig. S3B), suggesting normal physiological function. Thus, LHB is specifically required for postnatal mammary gland stages (puberty, pregnancy) that are characterized by massive, stem cell-driven tissue growth.

To assess whether the LBH-dependent failure in epithelial growth was a result of decreased cell proliferation, mammary gland sections were immunostained for proliferation marker Ki67 (Fig. 2E). As expected, in pubertal wild-type glands the majority of Ki67 + cells (~60%) were detected in TEBs, which are enriched in actively proliferating MaSC/progenitor cells (Bai and Rohrschneider, 2010). Conversely, the ducts contained less than 6% of Ki67 + cells (Fig. 2E,F). In K14Cre;LhbloxP/loxP mutant glands, the number of Ki67 + cells in the ducts was the same as in wild type; however, the number of Ki67 + basal and luminal cells in TEBs was significantly reduced (>25%; P < 0.01) (Fig. 2E,F). Reduced TEB cell proliferation was not due to increased apoptosis because immunostaining of adjacent sections for activated caspase 3 detected an equal number (<1%) of positive cells in mammary glands of both genotypes (data not shown). Moreover, a marked reduction in Ki67 + proliferating alveolar cells was observed in pregnant K14Cre;LhbloxP/loxP glands (supplementary material Fig. S3C), further supporting the notion that the mammary gland outgrowth defects caused by loss of LBH likely resulted from impaired stem/progenitor cell expansion.

Loss of Lhb results in abnormal mammary epithelial cell morphology and lineage differentiation

As ubiquitous Lhb-null mice, which we previously generated though breeding of LhbloxP/loxP mice with a ROSA26-Cre (R26-Cre) deleter strain (Lindley and Briegel, 2013), are viable and exhibit mammary gland outgrowth defects similar to K14Cre;LhbloxP/loxP mutants, these mice were included in the subsequent analyses. Histological analysis revealed that overall the bi-layered structure of mammary glands from K14Cre;LhbloxP/loxP and R26Cre;LhbloxP/loxP mice was intact, with an inner layer of luminal cells surrounded by an outer layer of baso-myoepithelial cells (Fig. 3A; supplementary material Fig. S4A). However, whereas in wild type (K14Cre;LhbloxP/loxP and R26Cre;LhbloxP/loxP), basal cells had a typical flat, spindle-like appearance, many cells in the basal layer of Lhb-deficient glands exhibited an abnormal cuboidal, more polarized epithelial morphology. Furthermore, the luminal cell layer in Lhb-deficient mammary glands was abnormally thickened and disorganized (Fig. 3A; supplementary material Fig. S4A). To assess whether these Lhb loss-of-function phenotypes were due to perturbed basal-luminal cell specification and/or differentiation, immunohistochemical analysis of lineage marker expression was performed.

Although expression of K5 was virtually unchanged in Lhb-deficient mammary glands in both Cre deleter backgrounds, indicating normal luminal lineage specification, expression of K5 was visibly reduced in the basal epithelium of Lhb mutant glands (Fig. 3B; supplementary material Fig. S4B). Furthermore, hormone receptor ERα and its target gene progesterone receptor (PR; Pgr – Mouse Genome Informatics) were expressed in far greater numbers of luminal cells (60% and 55% more, respectively) and with increased immunostaining intensity in Lhb-deficient glands relative to wild type, which typically express ERα/PR in ~30% of luminal cells (Fig. 3C,D; supplementary material Fig. S4C). Serum estradiol concentrations were normal in K14Cre;LhbloxP/loxP mutant mice (Fig. 3E), indicating that the aberrant luminal overexpression and increased transcriptional activity of ERα, as reflected by expression of PR, and the delayed ductal elongation in pubertal Lhb knockout mice were not due to changes in systemic hormone levels.
To quantify the LBH-dependent changes in basal-luminal marker expression, qPCR analysis of FACS-purified MEC subpopulations was performed. Consistent with our immunohistochemistry protein data, mRNA levels of K5 and stem cell marker Axin2, were significantly reduced in basal subpopulations (by 60-70% and 50-60%, respectively; P<0.01). By contrast, expression of the luminal differentiation marker ERα was profoundly upregulated (>four- to ninefold; P<0.01) in luminal cell fractions from K14-Cre; LbhloxP/loxP and R26Cre;LbhloxP/loxP mice (Fig. 3F; supplementary material Fig. S4D). We also observed a slight increase in K8 levels in luminal populations of Lbh-deficient mice; however, this increase was significant only in K14-Cre; LbhloxP/loxP mice (Fig. 3F).

Analysis of lineage-specific transcription factors (TFs) in K14-Cre;LbhloxP/loxP mammary glands further showed that, surprisingly, the expression levels and patterns of SLUG and GATA3, which are known to control basal or luminal cell determination, respectively (Asselin-Labat et al., 2007; Guo et al., 2012; Kouros-Mehr et al., 2006), were not significantly changed (Fig. 4A,B). However, the stem cell-specific isoform of p63, ΔNp63, was profoundly downregulated at the mRNA level (~60%) and absent at the protein level in Lbh-deficient basal cells (Fig. 4A,B), whereas its luminal-specific isoform, TAp63, was drastically upregulated (>400%) in luminal cells of K14-Cre;LbhloxP/loxP glands compared with wild type (Fig. 4A,B). Taken together, these data suggest that loss of Lbh specifically impairs the basal MaSC compartment and alters the differentiation status of luminal cells.

**Loss of Lbh decreases the frequency and activity of basal mammary stem cells**

To identify the cellular mechanisms underlying the mammary gland outgrowth defects and lineage imbalances observed in Lbh-deficient mice, we next asked whether MaSC/progenitor cell function was perturbed. FACS analysis revealed a striking reduction in the basal MaSC-containing CD29hiCD24lo population (~35%; P<0.05) in both K14-Cre;LbhloxP/loxP and R26Cre;LbhloxP/loxP mice (Fig. 5A,B; supplementary material Fig. S5A). By contrast, the luminal CD29hiCD24lo subpopulation was slightly but significantly increased (+17%; P<0.05). To measure stem/progenitor cell activity, primary MECs from Lbh-deficient and wild-type mice were plated as single cell suspensions in non-adherent mammosphere suspension cultures. In this in vitro assay, both unipotent and multipotent progenitor cell lines can form primary spheres, but only stem cells with increased self-renewal potential are capable of efficient sphere formation after serial passaging (Dontu et al., 2003). We observed ~50% reduction in sphere formation (P<0.01) for unsorted Lbh-deficient MECs (Fig. 5C; supplementary material Fig. S5B), indicative of overall reduced stem/progenitor cell activity. Subsequent sphere assays with FACS-sorted epithelial subpopulations revealed that the diminished stem cell activity of Lbh-deficient MECs was due to a deficiency in basal MaSCs: whereas luminal CD29hiCD24lo populations from Lbh-deficient mice showed normal primary sphere formation, Lbh-deficient basal CD29hiCD24lo fractions gave rise to 70%
fewer primary spheres than the same cell populations from wild-type mice (Fig. 5D; supplementary material Fig. S5C). Furthermore, whereas wild-type basal cells exhibited a mammosphere repopulation ratio (MRR) of 80% relative to luminal cells after serial passaging, indicative of the pronounced self-renewal potential of this population, the MRR for basal cells derived from K14-Cre; LbhloxP/loxP glands was significantly reduced by 50% (P<0.05) (Fig. 5E). Collectively, these results demonstrate that loss of LBH affects the in vitro self-renewal potential of basal MaSCs, but does not significantly alter luminal stem/progenitor cell activity.

The differentiation potential of Lbh-deficient basal MaSCs is skewed towards the luminal lineage

To further ascertain that Lbh deficiency impaired the functionality of basal MaSCs, we assessed their unique multipotent lineage differentiation potential in vitro. FACS-purified CD29(lo)CD24(hi) basal populations from K14-Cre; LbhloxP/loxP, R26Cre; LbhloxP/loxP and respective wild-type mice were grown in mammosphere cultures for 14 days to enrich for MaSCs. Thereafter, individual primary spheres were plated on adherent collagen-coated culture slides to induce differentiation and lineage differentiation was assessed after 5 days by co-immunofluorescence staining for basal-luminal keratins (see Materials and methods). Under these conditions, spheres derived from wild-type basal cells gave rise to a majority (76-79%) of K5+/K8+ double-positive cells, which likely represent uncommitted progenitor cells (Smith et al., 1990). 6-7% of K5+ single-positive basal myoepithelial cells and 15-16% of K8+ single-positive luminal cells (Fig. 5F,G; supplementary material Fig. S5D,E). By contrast, spheres derived from K14-Cre; LbhloxP/loxP or R26Cre; LbhloxP/loxP basal cells gave rise to significantly reduced numbers of K5+/K8+ progenitors (~65% or ~57%, respectively; P<0.05) and showed a sharp decline in mature K5+ basal myoepithelial cells (from 6.1 to 2.3%; P<0.05; and from 7.3 to 1%; P<0.01, respectively). Conversely, the number of K8+ luminal cells was increased by over 120-160% compared with wild-type spheres (Fig. 5F,G; supplementary material Fig. S5D,E). These data indicate that Lbh-deficient CD29(lo)CD24(hi) basal subpopulations have reduced multipotency and are shifted in their differentiation potential towards a luminal cell fate at the expense of baso-myoepithelial cell specification.

Loss of LBH results in precocious luminal cell differentiation

The elevated numbers of ERα-expressing luminal cells in Lbh-deficient mammary epithelia in situ further raised the possibility that LBH inactivation may have caused more pronounced luminal cell differentiation. To test this, we segregated luminal cells of 8- to 10-week-old K14-Cre; LbhloxP/loxP knockout mice and K14-Cre; Lbh+/-loxP control littermates into individual ERα mature luminal (ML), as well as immature ERα− and ERα− luminal progenitor (LP) cell populations, using FACS double-sorting with sets of EpCAM-CD49f and Sca1-CD49b antibodies (Shehata...
et al., 2012) (Fig. 6). qPCR analyses confirmed that within the luminal Lin−/EpCAMhi/CD49flow fraction, the Sca1+CD49b− ML population exhibited the highest Erα levels. By contrast, Sca1+CD49b+ LPs expressed Erαmedium and Sca1−CD49b+ LPs expressed Erαlow levels, indicating they represent distinct Er+ and Er− luminal progenitor populations (Fig. 6A,B). Strikingly, although the distribution of Erα−/medium LPs (Sca1−CD49b−) was not significantly changed in K14-Cre;LbhloxP/loxP mutants, the Erαhigh ML (Sca1−CD49b−) cell population was abnormally increased (60% versus 41%; P = 0.01) (Fig. 6C,D). Conversely, the Erα−/low (Sca1−CD49b+) LP population, which likely represents immature alveolar progenitor cells (Shehata et al., 2012), was reduced by approximately the same amount (28% versus 46%; P = 0.003) (Fig. 6D). Although expression of lactogenic markers was not consistently increased in the expanded Erαhigh ML population at virgin stages (data not shown), pregnant Lbh−deficient mammary glands exhibited signs of precocious lactogenic differentiation, as evidenced by the abundant presence of milk protein and droplets in alveoli of K14-Cre;LbhloxP/loxP glands at day 12.5 of pregnancy (supplementary material Fig. S3A,C). Thus, loss of LBH prematurely induces terminal luminal differentiation.

**LBH induces ΔNp63, represses ERα and promotes ‘stemness’ of mammary epithelial cells**

Our in vivo and ex vivo studies thus far suggest that LBH is required to maintain a basal MaSC state, whereas it represses luminal epithelial differentiation. The specific changes in the expression levels of p63 isoforms and Erα, with basal ΔNp63 being downregulated and luminal TAp63 and Erα being upregulated in Lbh−deficient mammary glands (Figs 3C,F and 4A,B), led us to hypothesize that these lineage-specific TFs might play a role in LBH-dependent stem cell regulation and differentiation. We, therefore, examined regulation of p63 and Erα by LBH further in MEC culture systems. RNAi was used to efficiently deplete LBH expression in two normal-derived human MEC lines, MCF10A and 226L, which express endogenous LBH at low or high levels, respectively (Fig. 7A). Both of these cell lines exhibit basal characteristics and are Erαlow. Conversely, LBH was stably introduced into murine HC11 (Rieger et al., 2010) (Fig. 7D), one of the few normal MEC lines with luminal characteristics and endogenous Erα expression (Faulds et al., 2004).

When LBH was depleted in MCF10A and 226L cells by transient transfection with LBH-specific siRNAs, these cells displayed severely reduced mammosphere formation (−80%; P < 0.001) compared with control scrambled siRNA-transfected cells (Fig. 7B). By contrast, gain of function of LBH in HC11 significantly increased sphere formation by 50% (P < 0.05) (Fig. 7E). Mirroring these positive effects of LBH on stemness, ΔNp63 was significantly downregulated in MCF10A and 226L cells upon LBH knockdown (by 40% and 50%, respectively; P < 0.05), whereas it was upregulated (+130%; P < 0.05) in HC11 ectopically expressing LBH (Fig. 7C,F). TAp63 was not expressed at detectable levels in these MEC systems (data not shown) and, hence, could not be further analyzed. However, mRNA expression of Erα was significantly elevated (+30%) in LBH-depleted MCF10A and 226L cells, whereas both Erα mRNA and protein levels were markedly downregulated (−50%) in LBH-expressing HC11 versus vector-expressing cells (Figs 7F and 8D). These data suggest that LBH normally promotes stemness of MECs and does so mechanistically by inducing the basal MaSC-specific TF ΔNp63, while it represses Erα, which is essential for luminal cell proliferation and differentiation (Mallepell et al., 2006).

**ΔNp63 acts downstream of LBH in promoting a basal MaSC phenotype and in Erα repression**

As ΔNp63 has recently been shown to promote an immature basal MaSC state (Li et al., 2008; Yalcin-Ozuysal et al., 2010), we next investigated whether ΔNp63 could play a role in LBH-induced stemness of MECs. HC11-vector- and HC11-LBH-expressing cells were transiently transfected with siRNAs specific to p63. Efficient knockdown of ΔNp63 was confirmed by qPCR and western blot analyses, respectively (Fig. 8A,D). Furthermore, qPCR was used to ascertain that ΔNp63 knockdown did not affect exogenous Lbh expression in these cells (Fig. 8A). Remarkably, depletion of ΔNp63 abolished the increase in sphere formation observed for LBH-overexpressing HC11 cells (Figs 7E and 8B). Moreover, lineage-specific gene expression was changed: while ΔNp63 knockdown led to the de-repression of luminal Erα in LBH-expressing HC11
cells, it concomitantly reduced expression of basal K5, which was elevated in HC11-LBH relative to control HC11-vector cells (Fig. 8C,D). By contrast, expression levels of K8, which were modestly decreased in HC11-LBH cells, did not significantly change (Fig. 8C,D). These results suggest that ΔNp63 is required for both the stem cell promoting effects of LBH, as well as for LBH-mediated repression of ERα.

**DISCUSSION**

In this study, we have identified the first essential physiological role of the WNT-controlled transcription co-factor LBH as a crucial regulator of adult breast stem cells and epithelial lineage differentiation. Our results support a model (Fig. 8E) in which LBH promotes the replicative potential of basal MaSCs through repression of ERα. In this regard, it is striking that K14-Cre;LbhloxP/loxP mutant mice exhibited a drastic reduction in both the frequency and differentiation potential of basal MaSCs.

**Fig. 5. Loss of Lbh reduces the frequency, activity and differentiation potential of basal MaSCs.** (A) Representative FACS dot plots depicting the distribution of luminal and basal subpopulations within the Lin^− (Lin: CD45/CD31/TER119) cell fraction of K14-Cre;Lbh^+^ wild-type and K14-Cre;Lbh^loxP/loxP^ mutant glands at 8 weeks of age. (B) Quantification of the FACS reporter line (Bai and Rohrschneider, 2010) suggest that activated luminal stem cells alone or multi-potent basal MaSCs generate mammary gland epithelium in vivo (van Amerongen et al., 2012; Van Keymeulen et al., 2011; Prater et al., 2014; Rios et al., 2014), in situ stem cell marker analyses (Williams and Daniel, 1983) and in vivo labeling of activated MaSCs using a transgenic SHIP-GFP reporter line (Bai and Rohrschneider, 2010) suggest that activated MaSCs have basal characteristics, including a CD29^+^CD24^−^ marker profile. Moreover, tracing of WNT-responsive MaSCs using Lgr5 or Axin2 gene promoters has indicated that these MaSCs are multi-potent basal cells, contributing to both basal and luminal lineages in a clonal fashion (Rios et al., 2014; van Amerongen et al., 2012). In this regard, it is striking that K14-Cre;LbhloxP/loxP mutant mice exhibited a drastic reduction in both the frequency and in vitro self-renewal activity of CD29^+^CD24^−^ basal cells at late puberty, whereas the luminal CD29^+^CD24^−^ population was actually increased and luminal progenitor activity was normal, based on our primary mammosphere assays (Fig. 5D,E). In vitro differentiation assays, showing that purified Lhb-deficient basal MaSCs cells had an increased propensity to differentiate into K5^+^ luminal epithelial cells at the expense of basal/myoepithelial cell differentiation (Fig. 5E,F), further support these lineage imbalances may be due to dysfunctional basal MaSC differentiation with a bias towards the luminal lineage. Interestingly, these cellular abnormalities were the same in R26-Cre;LbhloxP/loxP mice, which lack LBH expression in all cells, including stromal cells (Lindley and Briegel, 2013), indicating that stromal LBH does not have a
role in MaSC regulation. From these results, and given that in vivo gain of function of LBH in embryonic tissues promoted a proliferative, undifferentiated progenitor state (Briegel et al., 2005; Conen et al., 2009), we conclude that LBH plays an essential, cell-autonomous role in the expansion and/or maintenance of multipotent basal MaSCs, as well as in mammary epithelial lineage specification.

The phenotypes of Lbh-deficient mice further indicate that LBH is required for proper basal-luminal cell differentiation. The basal layer, and/or isolated basal cell populations, from Lbh-deficient glands showed quantitative reductions in K5 levels, suggestive of an overall reduction in basal characteristics. Additionally, changes in basal cell morphology with a more polarized cell structure suggest LBH regulates cell adhesion. By contrast, the luminal epithelium was abnormally thickened and contained approximately twice as many ERα+/PRα+ luminal cells as wild-type glands, which we showed were mature ERα+/high luminal cells, forming a second outer luminal cell layer. Normal percentages of ERα+/medium LPs and lack of tumor formation in aged K14-Cre;LbhloxP/loxP and R26-Cre; LbhloxP/loxP mice (data not shown) further rule out hyperplasia as a cause for the abnormal luminal thickening. Furthermore, epithelial-specific LBH ablation induced precocious lactogenic differentiation, which is consistent with our previous finding that LBH gain of function in HC11 MECs blocks lactogenic differentiation (Rieger et al., 2010).

The mammary gland phenotypes of Lbh-deficient mice are strikingly similar to those of mice deficient in other WNT pathway genes (e.g. Lrp5, Pygo2), which also exhibit pubertal and parity-induced mammary outgrowth defects that are accompanied by reduced basal MaSC function and number, and a distorted basal-to-luminal cell ratio (Badders et al., 2009; Gu et al., 2009), with a skewing towards luminal alveolar differentiation (Gu et al., 2013). However, defects in basal cell morphology, luminal cell organization or an abnormal overexpression of hormone receptors (ERα, PR) have not been noted in these other WNT pathway mutants, suggesting LBH may have both WNT-dependent and WNT-independent functions. Moreover, the mammary gland defects in Lbh-deficient mice are distinct from those elicited by inactivation of other basal MaSC-promoting TFs, e.g. knockout of SLUG in mice did not affect pubertal outgrowth, although it reduced basal cell morphology, luminal cell signaling and LBH function, and another that drives tissue homeostasis and growth at late virgin stages.

Importantly, our work provides circumstantial but strong evidence that LBH acts upstream of p63, a TF in the p53 family (Yang et al., 1998) that is essential for epithelial morphogenesis.
expression in HC11-LBH cells upon mediated regulation of MaSCs and epithelial lineage differentiation. LBH normally loading control. Asterisks indicate non-specific bands. (E) Model for LBH-increased sphere formation observed for HC11-LBH cells; **P<0.01; ***P<0.001 (n=3). (D) Western blot analysis of total cell extracts from HC11-vector and HC11-LBH cells 3 days after transfection with scrambled control (siCtrl) or p63-specific (sip63) siRNAs. Values were normalized to Gapdh and represent means±s.e.m. (n=3). (B) Mammosphere assays showing that ΔNp63 knockdown abrogates the increased sphere formation observed for HC11-LBH cells; ***P<0.001 (n=3). (C) qPCR analysis reveals a de-repression of ERα and reduced basal K5 expression in HC11-LBH cells upon ΔNp63 knockdown; *P<0.05; **P<0.01; ***P<0.001 (n=3). (D) Western blot analysis of total cell extracts from HC11-vector (V) and HC11-LBH (L) cells 3 days post p63 siRNA transfection. Actin served as a loading control. Asterisks indicate non-specific bands. (E) Model for LBH-mediated regulation of MaSCs and epithelial lineage differentiation. LBH normally acts upstream of ΔNp63 to promote a basal MaSC state, and to repress luminal differentiation and ERα expression. In LBH-deficient mice, basal ΔNp63 expression is lost, resulting in decimation of basal MaSCs and an abnormal increase in differentiated ERα-high luminal cells.

Fig. 8. ΔNp63 is required for LBH-induced ‘stemness’ and repression of ERα. (A) qPCR analysis of ΔNp63 and Lbh expression in HC11-vector and HC11-LBH cells 3 days after transfection with scrambled control (siCtrl) or p63-specific (sip63) siRNAs. Values were normalized to Gapdh and represent means±s.e.m. (n=3). (B) Mammosphere assays showing that ΔNp63 knockdown abrogates the increased sphere formation observed for HC11-LBH cells; ***P<0.001 (n=3). (C) qPCR analysis reveals a de-repression of ERα and reduced basal K5 expression in HC11-LBH cells upon ΔNp63 knockdown; *P<0.05; **P<0.01; ***P<0.001 (n=3). (D) Western blot analysis of total cell extracts from HC11-vector (V) and HC11-LBH (L) cells 3 days post p63 siRNA transfection. Actin served as a loading control. Asterisks indicate non-specific bands. (E) Model for LBH-mediated regulation of MaSCs and epithelial lineage differentiation. LBH normally acts upstream of ΔNp63 to promote a basal MaSC state, and to repress luminal differentiation and ERα expression. In LBH-deficient mice, basal ΔNp63 expression is lost, resulting in decimation of basal MaSCs and an abnormal increase in differentiated ERα-high luminal cells.

(Mills et al., 1999; Yang et al., 1999). Differential promoter use produces two p63 isoforms, TAp63 and ΔNp63, which have the same DNA-binding domain but either contain or lack the N-terminal transactivation domain (Yang et al., 1998). In the mammary gland, TAp63 is specifically expressed in luminal cells (Li et al., 2008; Nylander et al., 2002) and is involved in the differentiation of MaSCs into luminal progenitor cells (Li et al., 2008). By contrast, ΔNp63 is expressed in basal MaSCs (Li et al., 2008; Lim et al., 2010) and is the major p63 isoform required for maintaining the replicative potential of basally located stem cells in epidermal tissues (Senoo et al., 2007; Yang et al., 1999). Strikingly, although ΔNp63 expression in the basal epithelial layer was abolished in K14-Cre::LbhloxP/loxP mice, TAp63 was drastically elevated in luminal cells, as well as abnormally in basal epithelial populations (Fig. 4A,B), suggesting that LBH normally induces ΔNp63, but represses TAp63 in MECs.

Downregulation of ΔNp63 in Lbh-deficient MaSCs could be responsible for a range of phenotypes observed in our model. ΔNp63 controls stem cell self-renewal and quiescence, and promotes basal epithelial characteristics by regulating transcription of genes involved in cell proliferation, adhesion and the basal cytoskeleton, including K5 (Carroll et al., 2006; Romano et al., 2009; Wu et al., 2003; Yalcin-Ozysal et al., 2010), which we found to be induced by LBH in HC11. By modulating LBH and p63 expression in established MEC culture systems, we demonstrate that LBH induces ΔNp63 at the mRNA level and that ΔNp63, indeed, acts downstream of LBH in promoting stemness and basal K5 expression. Moreover, ΔNp63 is required, at least in part, for LBH-mediated repression of ERα. Although the potential repression of TAp63 by LBH could not be further corroborated in vitro, elevated luminal TAp63 expression in Lbh-deficient mice coinciding with increased luminal differentiation, suggests that LBH could be repressing MaSCs differentiation into luminal cells through repression of TAp63. Intriguingly, recent functional studies using primary mouse and human MECs have suggested that the ΔNp63 and TAp63 expression ratio may act as an important genetic switch that determines basal MaSC regeneration versus non-regenerative differentiation into luminal progenitors (Li et al., 2008; Yalcin-Ozysal et al., 2010). As LBH possesses both transcriptional co-activator and co-repressor activity (Briegel et al., 2005; Briegel and Joyner, 2001), it is plausible that LBH activates ΔNp63 and represses TAp63 directly at the promoter level. Alternatively, LBH may regulate signaling pathways, such as Hedgehog or Notch, which have been shown to suppress ΔNp63, but induce TAp63 to promote luminal progenitor specification (Li et al., 2008; Yalcin-Ozysal et al., 2010). Future experiments will be needed to resolve these intriguing possibilities.

Collectively, our results highlight LBH as a novel master regulator of breast epithelial lineage determination that acts upstream of ΔNp63 to promote a multipotent basal MaSC state and repress luminal differentiation. These findings are highly significant given that in human breast cancer, LBH is aberrantly overexpressed in the most lethal basal tumor subtype (Lamb et al., 2013; Rieger et al., 2010), which is hormone receptor negative and enriched in cancer stem cells (Honeth et al., 2008; Sorlie et al., 2003). Future studies will be necessary to elucidate the precise mechanism by which LBH regulates basal MaSC function and its role in carcinogenesis, as well as to determine its contribution to the stem cell-promoting effects of WNT in normal and neoplastic mammary gland development.

MATERIALS AND METHODS

Mice

Conditional LbhloxP knock-out mice were generated in a 129/SvEv×C57BL/6 genetic background and genotyped, as described previously (Lindley and Briegel, 2013). K14-Cre::Tg(KRT14-cre)1Amc/J) mice (Bassist et al., 2000) and Lgr5-GFP-CreERT2 [B6.129P2-Lgr5tm1(cre/ERT2)Cle/J; stock #008875] mice (Barker et al., 2007) were from The Jackson Laboratory, and ROSA26-Cre mice (Otto et al., 2009) were from Taconic-Artems [C57BL/6-Tac-Cre (ROSA26)tm16CreosCreer, stock #6467]. All studies were approved by the University of Miami IACUC committee.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Lindley and Briegel, 2013) using 5 μm sections of paraaffin-embedded abdominal mouse mammary glands fixed overnight in 4% paraformaldehyde or commercial FFPE human breast tissues (Biomax). Primary antibodies used were to LBH (1:75-100; affinity-purified; see methods in the supplementary material), keratin 5/6 (1:5000; Covance), keratin 8 (1:500; TraumaD; Hybridoma...
Flow cytometry and cell sorting
Mammary cells (2×10^6), isolated from 7- to 9-week-old females mice (see methods in the supplementary material), were blocked for 10 min in ice-cold PBS+2% HI-FBS containing anti-CD16/CD32 (Invitrogen) and anti-γ-globulin (Jackson ImmunoResearch) antibodies. Cells were immunostained for 30 min with AP-conjugated CD45, CD31 and TER119 antibodies (BD Biosciences) specific to Lineage (Lin) markers in combination with anti-CD24-PE (BD Biosciences) and anti-CD29-FITC (Serotec) antibodies for CD24-CD29 analyses (Shackleton et al., 2006), or with anti-EpCAM-FITC, anti-CD49f-Pacific Blue, anti-Scal-PE/Cy7 and anti-CD49b-PE antibodies (Biolegends) for luminal cell analyses (Shehata et al., 2012). Labeled cells were washed with ice-cold PBS+2% HI-FBS, incubated for 30 min with Streptavidin-APC (Invitrogen) and with violet dead cell marker (Invitrogen) to exclude Lin+ and dead cells, filtered through a 40 µm filter (BD Falcon), and sorted using a FACSAria-IIu (BD Biosciences) and FlowJo software.

Mammosphere assays
Single cell suspensions of primary MECs or MEC lines (see methods in the supplementary material) were plated in triplicate on poly HEMA (2-hydroxyethyl methacrylate) (Sigma) pre-coated six-well plates at a density of 1×10^5 cells or 5×10^4 cells per well, respectively, as described previously (Shaw et al., 2012). Cells were grown in mammosphere media [DMEM/F12 with Phenol Red, 20 ng/ml EGF, 20 ng/ml FGF, 4 µg/ml heparin, 1 mg/ml penicillin/streptomycin and B27 supplement (Invitrogen)] for 10-14 days (primary MECs) or 7 days (MEC lines) at 37°C in a 5% CO2 incubator. Spheres (>50 µm in diameter) were counted and quantified. For serial passaging, primary spheres were collected in culture media by centrifugation at 450 g for 5 min, washed with PBS, trypsinized and mechanically dissociated into a single cell suspension using a 25-gauge syringe. After washing in media containing 2% HI-FBS, cells were resuspended in PBS for counting and re-plating in secondary mammosphere cultures.

2D mammosphere differentiation assay
Primary mammospheres were transferred to collagen-coated eight-well chamber slides (BD) as described previously (Pei et al., 2009), grown in differentiation medium [HuMEC+supplements, 5% FBS (Invitrogen)] for 5 days in a 5% CO2 incubator at 37°C and assessed by immunofluorescence.

Quantitative real-time PCR
Total RNA was isolated using Trizol (Invitrogen) and qPCR analysis was performed as previously described (Lindley and Briegel, 2010) using iQ SYBR Green Supermix and a CFX96 Real Time PCR thermocycler (BioRad). Samples were assayed in triplicates and average Ct values were normalized to GAPDH. For qPCR primers, see Table S1 in the supplementary material.

Whole-mount analysis
Carmine Red whole-mount staining of inguinal murine mammary glands at different postnatal stages was performed as previously described (Lindley and Briegel, 2013).

 Estradiol measurements
Blood was collected by intracardiac puncture from 8-week-old anesthetized female mice. Serum was subjected to ERα-specific ELISA at the Virginia Center for Research in Reproduction Ligand Core Laboratory (Charlottesville, VA, USA).

Immunofluorescence
Differentiated mammosphere cultures were co-stained with antibodies to keratin 5/6 (1:1,000; Covance) and keratin 8 (1:400; Hybridoma Bank), followed by Alexa Fluor 488-594 secondary antibody (1:200; Invitrogen) incubations, as described previously (Lindley and Briegel, 2010).

Western blot analysis
Immunoblotting was performed as described previously (Rieger et al., 2010), using 20 µg of total cell extracts and antibodies to LBH (1:1,000; in house), keratin 5/6 (1:10,000; Covance), keratin 8/18 (1:2,000; Progen), ERα (1:1,000; HC-20, Santa Cruz), ΔNp63 (1:1000; Biolegend) or β-actin (1:10,000; AC-15; Sigma), as well as secondary HRP-conjugated IgGs (1:25,000; Invitrogen).

Statistical analyses
All statistical analyses were performed using unpaired two-tailed Student’s t-tests in Excel. P<0.05 was considered to be significant.

Author contributions
Conditional Lbh mice were designed by K.J.B. and generated by inGenious Targeting; generation and analysis of K14-Cre;LbhloxP and R26Cre;LbhloxP mice was carried by L.E.L. and K.J.B.; K.M.C. performed qPCR and M.E.R. carried out initial ERα expression analyses; A.S.-M. and D.J.R. provided reagents and assistance with primary MEC preparation; K.J.B. and L.E.L. wrote the manuscript.

Funding
This study was supported by grants from the Florida State Department of Health (05NIR-01-5186) and the National Institutes of Health/National Institute of General Medical Sciences (NIH/NIHGM5 [RO1GM113256] to K.J.B., by Department of Defense grants [W81XWH081053 (M.E.R.) and W81XWH1010430 (D.J.R.)], and by a NIH/National Institute of Arthritis and Musculoskeletal and Skin Diseases F32 post-doctoral fellowship [7F32AR062990-02] (K.M.C.). Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.110403/DC1

References


