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RESEARCH ARTICLE

STEM CELLS AND REGENERATION

The WNT-controlled transcriptional regulator LBH is required for mammary stem cell expansion and maintenance of the basal lineage

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ABSTRACT

The identification of multipotent mammary stem cells (MaSCs) has provided an explanation for the unique regenerative capacity of the mammary 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 mammopoiesis. We show that in postnatal mammary epithelia, LBH is predominantly expressed in the $Lin^{-}CD29^{high}CD24^{+}$ basal MaSC 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 ΔNp63 to promote a basal MaSC state and repress luminal differentiation genes, mainly that encoding estrogen receptor α (*Esr1*/*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 ($K8^{+}/K18^{+}$), and an outer layer of basal/myoepithelial cells, expressing basal keratins ($K5^{+}/K14^{+}$) (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 $CD29^{high}/CD49f^{high}/CD24^{+/mod}/Sca1^{-/low}$ surface marker profile, express a basal gene signature and are hormone-receptor negative (Asselin-Labat et al., 2006; Shackleton et al., 2006; Sleeman et al., 2006, 2007; Stingl et al., 2006). Lineage-tracing studies in mice have confirmed the existence of bi-potent basal MaSCs in the mammary 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 (Chaffer 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 (*XlCl2*) 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

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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 mammopoiesis 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 inner 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).

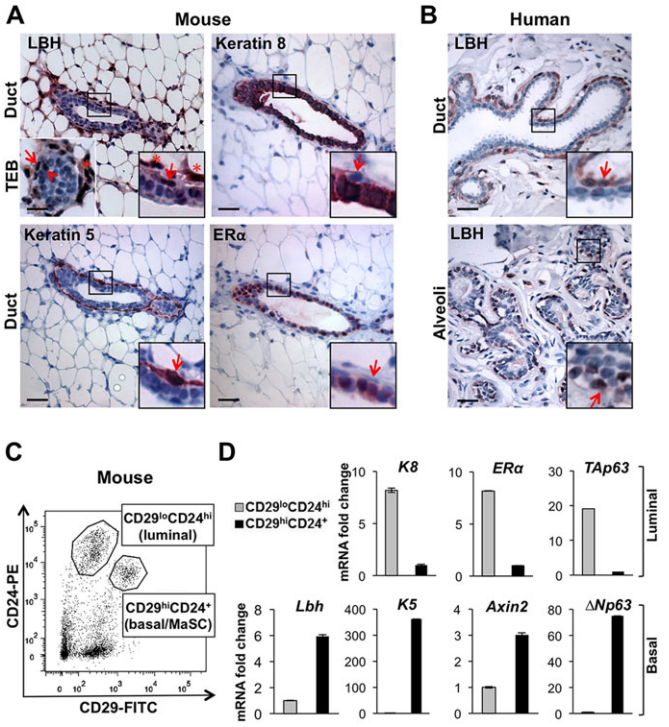

Fig. 1. LBH is predominantly expressed in basal mammary epithelial cells. (A) Immunohistochemical staining of serial mammary gland sections from 8-week-old virgin female mice with antibodies specific to LBH and to basal (keratin 5) and luminal (keratin 8, ERα) lineage markers. Left inset: LBH expression 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 (CD29^{lo}CD24^{hi}) and basal (CD29^{hi}CD24^{+}) 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.

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^{-}CD29^{low}CD24^{high} and basal Lin^{-}CD29^{high}CD24^{+} MEC populations (referred to as CD29^{lo}CD24^{hi} or CD29^{hi}CD24^{+}, 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).

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To further evaluate whether LBH is associated with WNT-responsive MaSCs, we examined *Lbh* 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$^+$ basal subpopulations (supplementary material Fig. S1A). As predicted, GFP$^+$ cells clustered within the CD24$^+$ 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, *Lbh* was highly enriched in the Lgr5-positive GFP$^+$CD24$^+$ 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 *Lbh* 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 Lbh impairs postnatal mammary gland development

We next used a loss-of-function strategy to elucidate the *in vivo* role of LBH in mammogenesis. We have previously generated mice with a conditional *Lbh* allele (*Lbh^{loxP}*), in which exon 2 of *Lbh* 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). *Lbh^{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 epithelia 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;Lbh$^{loxP/loxP}$* and control *K14Cre;Lbh$^{+/+}$* (referred to as wild type) glands showed that no functional *Lbh* mRNA was expressed in CD29^{hi}CD24$^+$ basal cells, its low-level expression in CD29^{lo}CD24^{hi} luminal cells was reduced; however, the abundant expression of *Lbh* in stromal (CD29^{lo}CD24^{lo}) cells was essentially unchanged in *Lbh* mutants compared with the respective cell populations of wild-type mice (Fig. 2A). Immunohistochemistry confirmed the absence of LBH protein in *K14Cre;Lbh$^{loxP/loxP}$* mammary epithelia, whereas LBH was still expressed in individual stromal cells (Fig. 2A,B).

Prior to puberty (4 weeks of age), *K14Cre;Lbh$^{loxP/loxP}$* glands were indistinguishable from wild-type glands, suggesting primordial mammogenesis 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 *Lbh* 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 *Lbh* 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;Lbh$^{loxP/loxP}$* females (supplementary material Fig. S3A). Nevertheless, lactation by *Lbh* mutant dams proceeded normally (supplementary material Fig. S3B), suggesting normal physiological function. Thus, LBH 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;Lbh$^{loxP/loxP}$* 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;Lbh$^{loxP/loxP}$* 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 *Lbh* results in abnormal mammary epithelial cell morphology and lineage differentiation

As ubiquitous *Lbh*-null mice, which we previously generated though breeding of *Lbh^{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;Lbh$^{loxP/loxP}$* mutants, these mice were included in the subsequent analyses. Histological analysis revealed that overall the bi-layer structure of mammary glands from *K14-Cre;Lbh$^{loxP/loxP}$* and *R26Cre;Lbh$^{loxP/loxP}$* 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 (*K14-Cre;Lbh$^{+/+}$* or *R26Cre;Lbh$^{+/+}$*), basal cells had a typical flat, spindle-like appearance, many cells in the basal layer of *Lbh*-deficient glands exhibited an abnormal cuboidal, more polarized epithelial morphology. Furthermore, the luminal cell layer in *Lbh*-deficient mammary glands was abnormally thickened and disorganized (Fig. 3A; supplementary material Fig. S4A). To assess whether these *Lbh* 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 K8 was virtually unchanged in *Lbh*-deficient mammary glands in both Cre deleter backgrounds, indicating normal luminal lineage specification, expression of K5 was visibly reduced in the basal epithelium of *Lbh* 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 *Lbh*-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 *K14-Cre;Lbh$^{loxP/loxP}$* 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 *Lbh* knockout mice were not due to changes in systemic hormone levels.

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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;Lbh*$^{loxP/loxP}$ and *R26Cre;Lbh*$^{loxP/loxP}$ 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;Lbh*$^{loxP/loxP}$ mice (Fig. 3F).

Analysis of lineage-specific transcription factors (TFs) in *K14-Cre;Lbh*$^{loxP/loxP}$ 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, Δ*Np63*, 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;Lbh*$^{loxP/loxP}$ 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 CD29^{hi}CD24^{+} population (−35%; $P<0.05$) in both *K14-Cre;Lbh*$^{loxP/loxP}$ and *R26Cre;Lbh*$^{loxP/loxP}$ mice (Fig. 5A,B; supplementary material Fig. S5A). By contrast, the luminal CD29^{lo}CD24^{hi} 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 cells 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 CD29^{lo}CD24^{hi} populations from *Lbh*-deficient mice showed normal primary sphere formation, *Lbh*-deficient basal CD29^{hi}CD24^{+} fractions gave rise to 70%

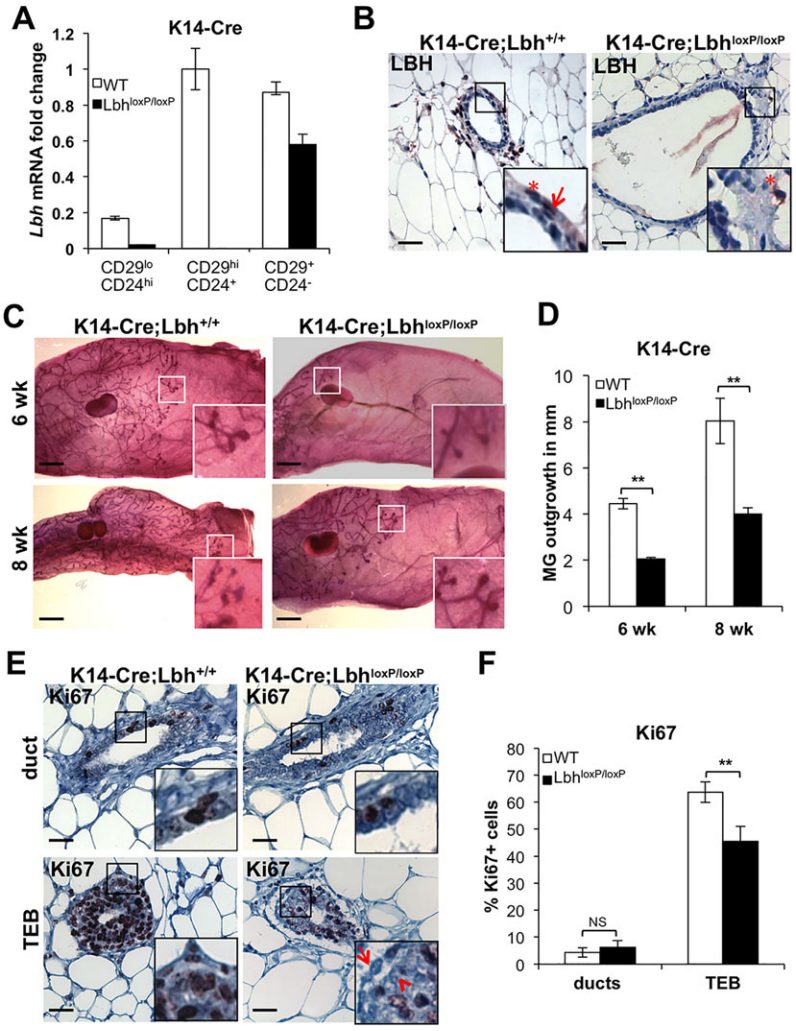

Fig. 2. Epithelial-specific *Lbh* inactivation delays pubertal mammary gland outgrowth. (A) qPCR analysis of *Lbh* expression in FACS-sorted luminal (CD29^{lo}CD24^{hi}), basal (CD29^{hi}CD24^{+}) and stromal (CD29^{+}CD24^{-}) populations from 8-week-old *K14-Cre;Lbh*$^{+/+}$ wild-type (WT) and *K14-Cre;Lbh*$^{loxP/loxP}$ glands. Values were normalized to *Gapdh* and represent mean±s.e.m. ($n=3$ mice per genotype). (B) Immunohistochemical staining of sections with anti-LBH antibody and higher magnifications thereof (insets). Basal cell-specific expression of LBH in wild type (arrow) and its stromal cell-specific expression in wild-type and *Lbh*-mutant glands (asterisks) are indicated. Scale bars: 25 µm. (C) Low- and high-power (insets) whole-mount images of pubertal glands from wild-type and *Lbh*-deficient mice at 6 and 8 weeks of age ($n\geq3$ each). Scale bars: 1 mm. (D) Quantification of mammary gland (MG) outgrowth into the fat pad, as measured in mm from the center of the lymph node. Values represent mean±s.d. ($n=3$); $^{**}P<0.01$. (E) Immunohistochemical staining of glands for Ki67 demonstrating reduced cell proliferation of basal (arrow) and luminal (arrowhead) cells in terminal end buds (TEBs) of *K14-Cre;Lbh*$^{loxP/loxP}$ mice. Scale bars: 40 µm. (F) Quantification of the percentage of Ki67-positive cells obtained by counting cells in three or more random sections per gland ($n=3$ per genotype). Values represent mean±s.d.; $^{**}P<0.01$; NS, non-significant.

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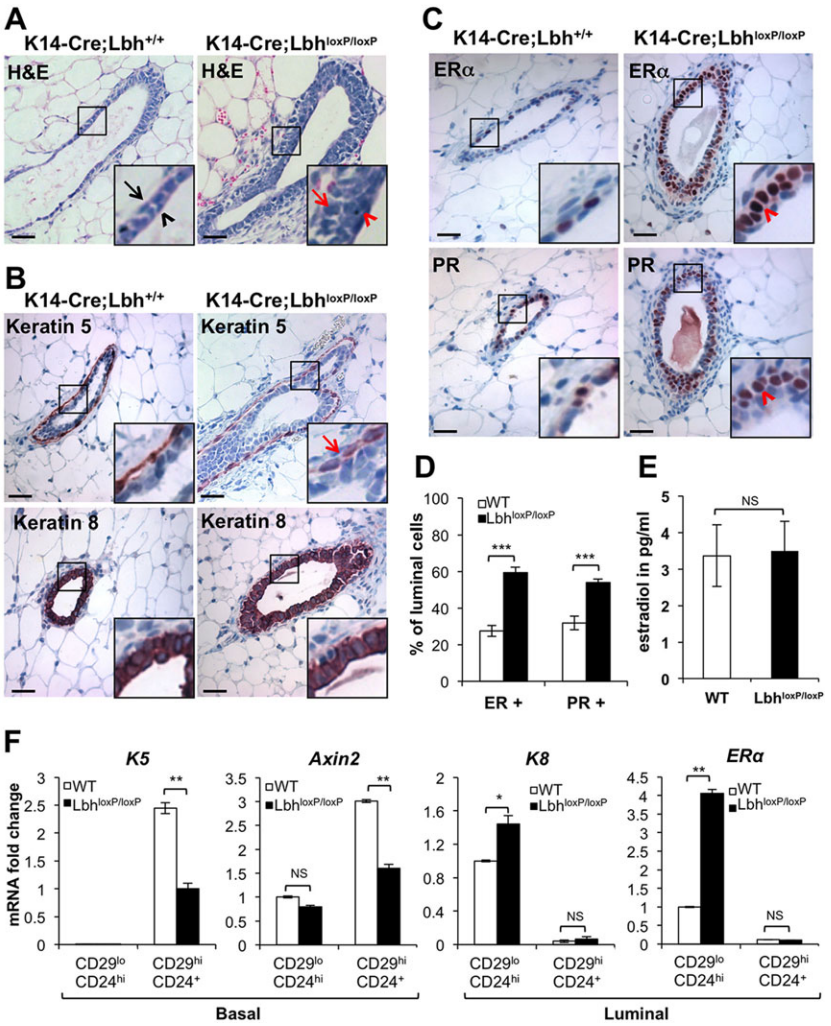

Fig. 3. Loss of *Lbh* impairs mammary epithelial cell morphology and lineage differentiation. (A) Hematoxylin and Eosin analysis of mammary gland sections from 8-week-old *K14-Cre;Lbh*$^{+/+}$ wild type (WT) and *K14-Cre;Lbh*$^{loxP/loxP}$ mice reveal aberrant basal cell morphology (red arrow) and thickening of the luminal epithelium (red arrowhead) in *K14-Cre;Lbh*$^{loxP/loxP}$ glands compared with wild type (black arrow indicates basal cells; black arrowhead indicates luminal cells). (B) Immunohistochemistry showing reduced basal keratin 5 expression (red arrow) in *K14-Cre;Lbh*$^{loxP/loxP}$ glands. (C) Immunohistochemistry showing an increased number of luminal cells (red arrowheads) positive for ERα and PR. (A-C) Insets contain higher magnifications of boxed areas. Scale bars: 25 µm. (D) Quantification of results in C (%). Values represent mean±s.d. (*n*=3 mice per genotype); ***P<0.001. (E) Serum estradiol levels (pg/ml) in 8-week-old females (*n*=3). (F) qPCR analysis of lineage markers in FACS-sorted luminal (CD29^{lo}CD24^{hi}) and basal (CD29^{hi}CD24^{+}) MEC populations. Values, normalized to *Gapdh*, represent the mean±s.e.m. (*n*=3); *P<0.05; **P<0.01; NS, non-significant.

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;Lbh*$^{loxP/loxP}$ 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^{hi}CD24^{+} basal populations from *K14-Cre;Lbh*$^{loxP/loxP}$, *R26Cre;Lbh*$^{loxP/loxP}$ 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;Lbh*$^{loxP/loxP}$ or *R26Cre:Lbh*$^{loxP/loxP}$ 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^{hi}CD24^{+} 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;Lbh*$^{loxP/loxP}$ knockout mice and *K14-Cre;Lbh*$^{+/loxP}$ control littermates into individual ER^{+} mature luminal (ML), as well as immature ER^{+} and ER^{-} luminal progenitor (LP) cell populations, using FACS double-sorting with sets of EpCAM-CD49f and Sca1-CD49b antibodies (Shehata

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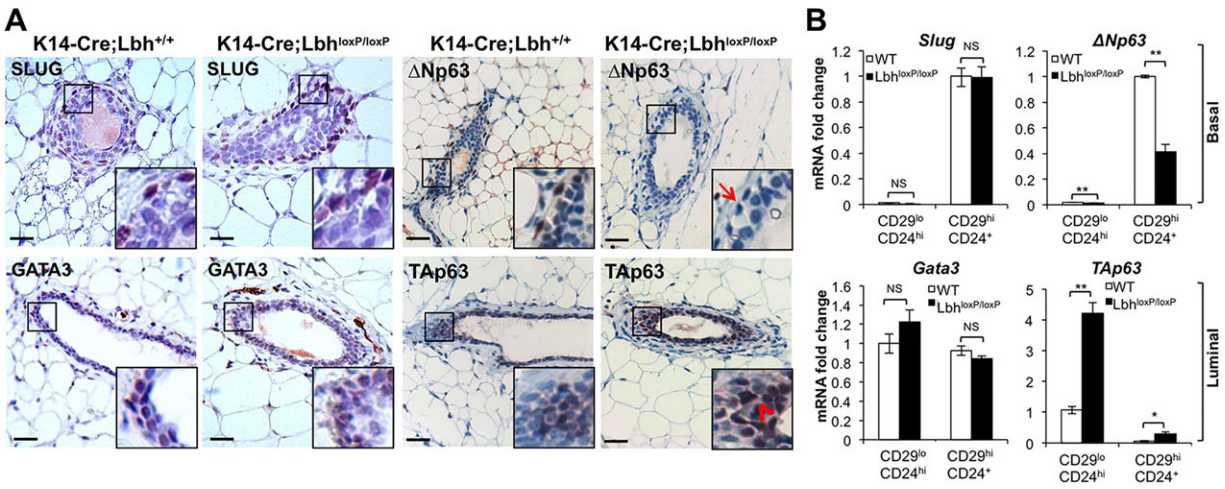

Fig. 4. *Lbh* ablation alters the expression of lineage-specific p63 isoforms. (A) Immunohistochemical analysis of basal (SLUG, ΔNp63) and luminal (GATA3, TAp63) lineage-specific TF expression in mammary gland sections from 8-week-old *K14-Cre;Lbh*$^{+/+}$ wild-type and *K14-Cre;Lbh*$^{loxP/loxP}$ mice. ΔNp63 protein is absent in basal epithelia (arrow) and there is abnormal overexpression of TAp63 in luminal cells (arrowhead) of *K14-Cre;Lbh*$^{loxP/loxP}$ glands. Insets contain higher magnifications of boxed areas. Scale bars: 25 µm. (B) qPCR quantification of results shown in A using FACS-purified luminal (CD29^{lo}CD24^{hi}) and basal (CD29^{hi}CD24^{+}) subpopulations. Values were normalized to *Gapdh* and represent the mean±s.e.m. (n=3); *P<0.05; **P<0.01; NS, non-significant.

et al., 2012) (Fig. 6). qPCR analyses confirmed that within the luminal Lin^{-}/EpCAM^{high}/CD49f^{low} fraction, the Sca^{+}CD49b^{-} ML population exhibited the highest *ERα* levels. By contrast, Sca1^{+}CD49b^{+} LPs expressed *ERα*$^{+/medium}$ and Sca^{-}CD49b^{+} LPs expressed *ERα*$^{-/low}$ 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;Lbh*$^{loxP/loxP}$ mutants, the ERα$^{+/high}$ ML (Sca^{+}CD49b^{-}) cell population was abnormally increased (60% versus 41%; P=0.01) (Fig. 6C,D). Conversely, the ERα$^{-/low}$ (Sca^{-}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 ERα$^{+/high}$ ML population at virgin stages (data not shown), pregnant *Lbh*-deficient mammary glands exhibited signs of precocious lactogenic differentiation, as evidenced by the abnormal presence of milk protein and droplets in alveoli of *K14-Cre;Lbh*$^{loxP/loxP}$ 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 ERα$^{-/low}$. 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

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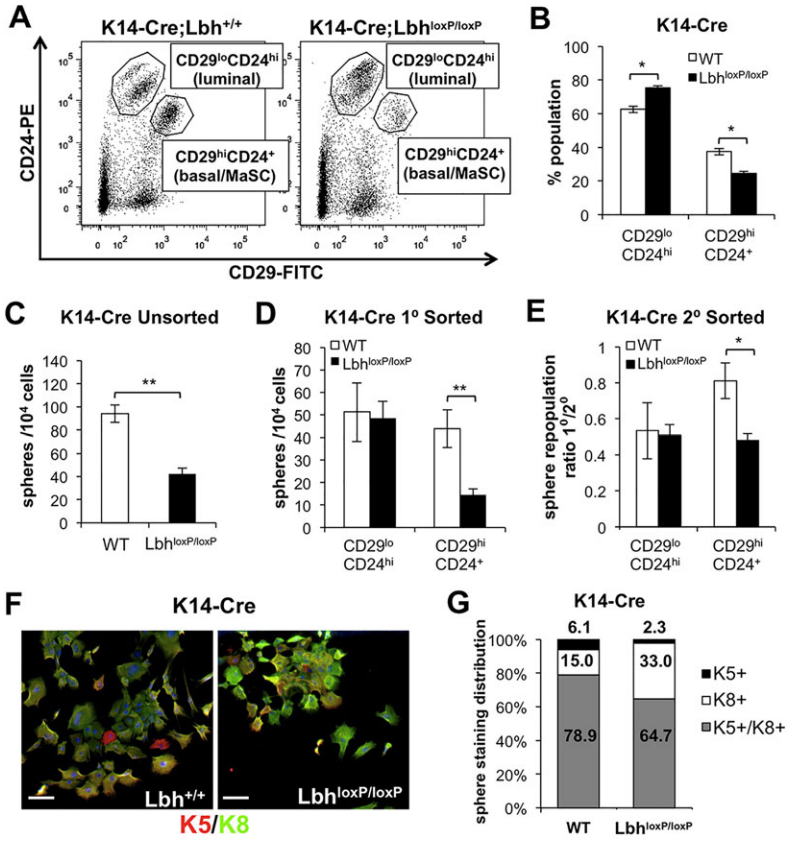

Fig. 5. Loss of *Lbh* reduces the frequency, activity and differentiation potential of basal MaSCs. (A) Representative FACS dot plots depicting the distribution of luminal and basal subpopulations within the Lin$^-$ (Lin: CD45/CD31/TER119) cell fraction of *K14-Cre;Lbh*$^{+/+}$ wild-type and *K14-Cre;Lbh*$^{loxP/loxP}$ mutant glands at 8 weeks of age. (B) Quantification of the FACS data in A in percent (%). (C-E) Mammosphere assays using: (C) unsorted primary MECs; (D) FACS-sorted luminal (CD29^{lo}CD24^{hi}) and basal (CD29^{hi}CD24^{+}) MEC populations; and (E) secondary cultures of dissociated primary spheres derived from individual luminal and basal subpopulations (see D). All values in A-E represent mean±s.d. (n=4 animals per genotype); *P<0.05; **P<0.01. (F) Co-immunofluorescent staining of differentiation-induced primary mammospheres derived from CD29^{hi}CD24^{+} basal/MaSC populations in D with antibodies to basal (K5; red) and luminal (K8; green) markers. Scale bars: 100 µm. (G) Histogram showing the percentages of K5^{+} basal (black), K8^{+} luminal (white) and K5^{+}/K8^{+} double-positive progenitor cells (gray) in basal cell-derived spheres 5 days after differentiation. Cells in 10 differentiated spheres from n=3 animals per genotype were counted. All differences between wild-type and *Lbh* mutant spheres were significant (P<0.05).

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 induction of epithelial stem cell TF ΔNp63. Furthermore, LBH, in a ΔNp63-dependent manner, maintains a basal MaSC state by repressing their non-regenerative elaboration into luminal progenitors and maturation into hormone receptor-positive cells through repression of ERα.

We first demonstrated that LBH is predominantly expressed, with a nuclear-specific pattern, in the regenerative basal epithelium of both mouse and human mammary glands, including in WNT-responsive Lgr5^{+} MaSCs. By contrast, LBH was not or was only barely expressed in luminal cells, which lack regenerative capacity in transplantation assays (Shackleton et al., 2006; Stingl et al., 2006) and multi-lineage differentiation potential *in situ* (van Amerongen et al., 2012; Van Keymeulen et al., 2011).

Importantly, we found that epithelial-specific, as well as ubiquitous, inactivation of *Lbh* in mice specifically disrupted postnatal mammary gland stages that require rapid MaSC expansion and differentiation (puberty and pregnancy). Reduced cell proliferation in TEBs and alveoli of pubertal or pregnant *K14-Cre;Lbh*$^{loxP/loxP}$ glands, respectively, further suggests that LBH is required for MaSC activation, as these lobular structures are normally enriched in actively proliferating MaSCs (Bai and Rohrschneider, 2010; Williams and Daniel, 1983) and express high levels of LBH (Kouros-Mehr and Werb, 2006; Rieger et al., 2010). Although it remains debatable whether unipotent basal and 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^{lo}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;Lbh*$^{loxP/loxP}$ mutant mice exhibited a drastic reduction in both the frequency and *in vitro* self-renewal activity of CD29^{hi}CD24^{+} basal cells at late puberty, whereas the luminal CD29^{lo}CD24^{hi} 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 *Lbh*-deficient basal MaSCs cells had an increased propensity to differentiate into K8^{+} luminal epithelial cells at the expense of basal/myoepithelial cell differentiation (Fig. 5E,F), further suggest 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;Lbh*$^{loxP/loxP}$ mice, which lack LBH expression in all cells, including stromal cells (Lindley and Briegel, 2013), indicating that stromal LBH does not have a

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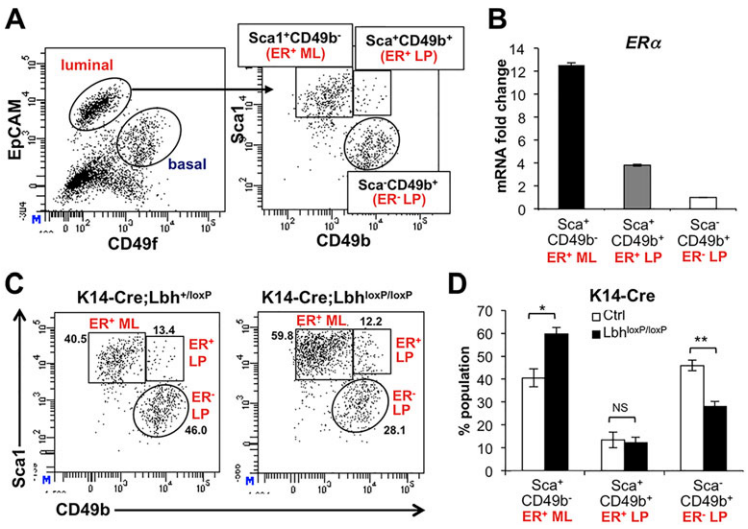

Fig. 6. Loss of *Lbh* increases luminal cell maturation. Luminal cells from 8- to 10-week-old virgin *K14-Cre;Lbh*$^{+/loxP}$ control (Ctrl) and *K14-Cre;Lbh*$^{loxP/loxP}$ knockout mice were segregated into ER^{+} mature luminal (ML), and ER^{+} or ER^{-} luminal progenitor (LP) populations by FACS using the Lin/EpCAM/CD49f/Sca1/CD49b surface marker set (Shehata et al., 2013). (A) Representative FACS plots showing the gating strategy. (B) qPCR quantification of *ERα* expression in control Lin^{-}/EpCAM^{hi}/CD49f^{lo} luminal subfractions normalized to *Gapdh*. (C) Representative Sca1/CD49b FACS plots showing reciprocal changes in the distribution of the ER^{+} ML and ER^{-} LP subpopulations in *Lbh*-deficient mice relative to controls. (D) Quantification of the results shown in C. All values represent the mean±s.d. (n=3 animals per genotype); *P<0.05; **P<0.01.

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^{+}/PR^{+} 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;Lbh*$^{loxP/loxP}$ and *R26-Cre;Lbh*$^{loxP/loxP}$ 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).

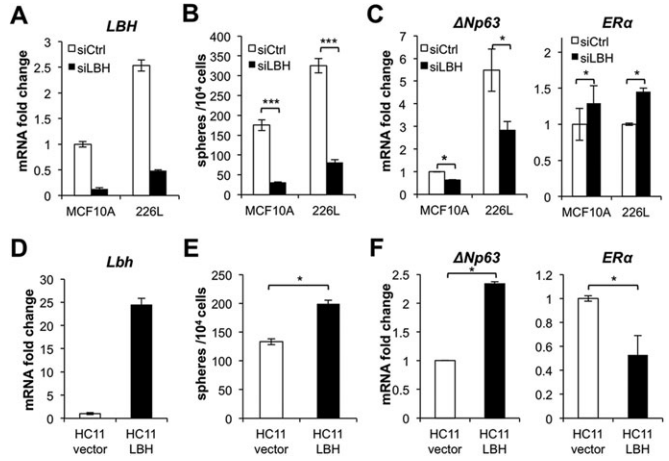

Fig. 7. LBH promotes 'stemness' and Δ*Np63* expression, while repressing ERα in MEC lines. (A) qPCR showing efficient depletion (>80%) of *LBH* mRNA in normal-derived human MCF10A and 226L MEC lines 3 days after transient transfection with LBH-specific siRNAs (siLBH). As a control, cells were transfected with scrambled siRNA (siCtrl). (B) Mammosphere assays using the same cells as in A show reduced sphere formation of siLBH-transfected cells. (C) qPCR detecting reduced Δ*Np63*, but increased *ERα* expression in both siLBH-transfected MCF10A and 226L cells. (D) qPCR analysis of *Lbh* expression in murine HC11 MECs stably transfected with empty pCDNA3 vector (HC11-vector) or a pCDNA3-LBH expression plasmid (HC11-LBH). (E) Mammosphere assays showing increased sphere formation of HC11-LBH cells. (F) qPCR detecting increased Δ*Np63*, but reduced *ERα* expression in HC11-LBH cells. All data represent mean±s.e.m. (n=3); *P<0.05; ***P<0.001.

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 MaSC function at late virgin stages (Phillips et al., 2014). Interestingly, *K14-Cre;Lbh*$^{loxP/loxP}$ mammary glands retained normal SLUG expression in basal cells, whereas other basal MaSC markers, *Axin2* and *ΔpN63*, were profoundly diminished. Thus, our work provides important novel insight into MaSC biology, as it suggests that different MaSC pools or states exist within the basal lineage (Prater et al., 2014; Rios et al., 2014): one that drives postnatal mammary gland outgrowth and requires WNT signaling and LBH function, and another that drives tissue homeostasis and requires SLUG, possibly through dedifferentiation of mature myoepithelial cells (Prater et al., 2014). Such a scenario could also explain why LBH-deficient glands eventually catch up with their 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

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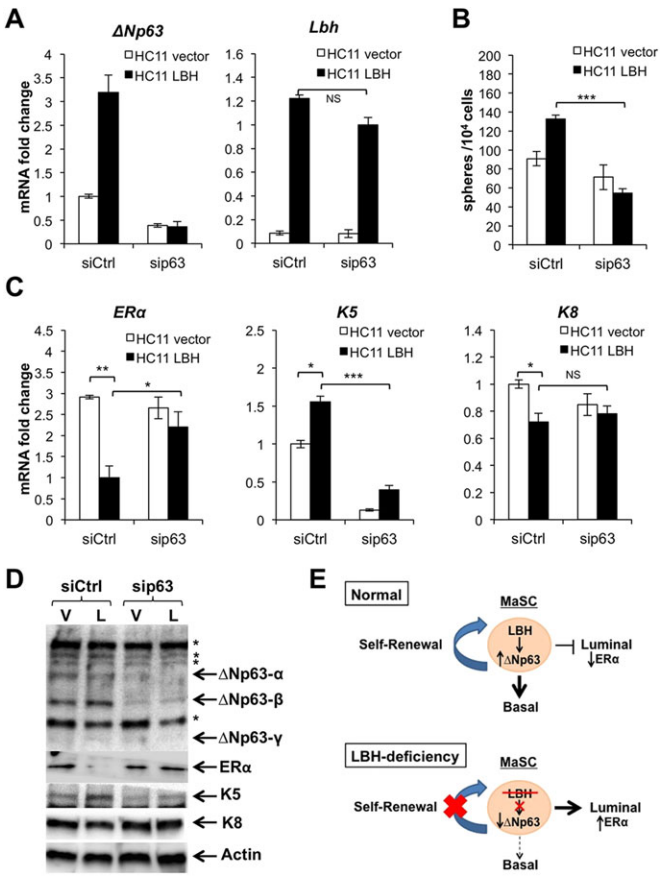

Fig. 8. Δ*Np63* is required for LBH-induced 'stemness' and repression of ERα. (A) qPCR analysis of Δ*Np63* and *Lbh* expression in HC11-vector and HC11-LBH cells 3 days after transfection with scrambled control (siCtrl) or p63-specific (sip63) siRNAs. Values were normalized to *Gapdh* and represent mean±s.e.m. (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;Lbh*$^{loxP/loxP}$ 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-Ozuysal 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-Ozuysal 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-Ozuysal 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 *Lbh*^{loxP} knockout 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; stock #004782] (Dassule et al., 2000) and *Lgr5-GFP-CreER*^{T2} [B6.129P2-*Lgr5*$^{tm1(cre/ERT2)Cle}$/J; stock #008875] mice (Barker et al., 2007) were from The Jackson Laboratory, and *ROSA26-Cre* mice (Otto et al., 2009) were from Taconic-Artemis [C57BL/6-Tac-*Gt(ROSA)26Sor*$^{tm16(Cre)Arte}$; 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 paraffin-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; TromaD; Hybridoma

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Bank), ERα (1:300; MC-20; SantaCruz), PR (1:300; C-19; Santa Cruz), Ki67 (1:300; Novus), ΔNp63 and TAp63 (1:500; Biolegend), and milk (1:1000; RAMT; Nordic), followed by incubation with HRP-coupled secondary antibodies (1:500; Invitrogen).

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 (BD Biosciences) and rat-γ-globulin (Jackson ImmunoResearch) antibodies. Cells were immunostained for 30 min with APC-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-Sca1-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 $1\text{-}2\times10^4$ cells or 5×10^3 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% CO_2 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% CO_2 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 (Bio Rad). 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conditional *Lbh* mice were designed by K.J.B. and generated by inGenious Targeting; generation and analysis of *K14-Cre;Lbh*^{loxP} and *R26Cre;Lbh*^{loxP} mice was carried out by L.E.L. and K.J.B.; K.M.C. performed p63 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.

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Supplementary material

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

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