Role of the WNT-Regulated Transcription Cofactor LBH in Normal and Neoplastic Mammary Stem Cell Control

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UNIVERSITY OF MIAMI

ROLE OF THE WNT-REGULATED TRANSCRIPTION COFACTOR LBH IN NORMAL AND NEOPLASTIC MAMMARY STEM CELL CONTROL

By

Linsey E. Lindley

A DISSERTATION

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ROLE OF THE WNT-REGULATED TRANSCRIPTION COFACTOR LBH IN
NORMAL AND NEOPLASTIC MAMMARY STEM CELL CONTROL

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Breast cancer (BC) is the second leading cause of cancer-related death in women with aggressive basal-subtype BCs contributing disproportionately to the rate of cancer deaths. Owing to a lack of hormone receptor and HER2 expression in basal-subtype BCs (also called ‘triple-negative’ BC or TNBC), no molecular targeted therapies are currently available to treat this lethal metastatic type of BC. Intriguingly, basal-subtype breast tumors are characterized by a highly undifferentiated tumor phenotype enriched in ‘cancer stem cells’ (CSCs), as well as hyperactivation of the oncogenic WNT signaling pathway. WNT plays a fundamental role in stem cell regulation during embryogenesis, adult tissue homeostasis, and carcinogenesis. Although targeting the WNT pathway represents a promising new therapy to eradicate treatment-refractory CSCs, no WNT-inhibiting drugs are currently in clinical use.

Previous work by our laboratory has identified a novel key WNT-controlled transcriptional cofactor LBH (Limb-Bud and Heart) with hitherto unknown physiological function. LBH represents a promising new molecular target for TNBC, as it is aberrantly overexpressed in basal-subtype breast tumors in a
WNT-dependent fashion. LBH, similar to other WNT pathway genes, is also expressed during normal mammary gland (MG) development. Using Fluorescence activated cell sorting (FACS) of primary mouse mammary epithelial cells (MEC), we show that LBH is restricted to the basal, hormone receptor-negative epithelial cell layer. This population of cells, contains multipotent stem cells that drive MG expansion during puberty and pregnancy and MG maintenance. This thesis, therefore, tested the hypothesis that LBH is implicated in both normal mammary stem cell (MaSC) development as well as in CSC formation during oncogenesis. The major aims were 1) to determine the mechanistic function of LBH in normal MG development using a novel conditional Lbh knockout mouse model; and 2) to ascertain the role of LBH in CSC formation by experimentally modulating LBH expression in human breast cancer cell lines.

Wholemount analysis showed that both global and epithelial-specific Lbh gene inactivation resulted in a pronounced delay in MG expansion during puberty and pregnancy. Additionally, Lbh-deficient MGs exhibited a depleted basal epithelial cell population, whereas the luminal cell layer was abnormally thickened and luminal expression of Estrogen Receptor alpha (ERα) and Progesterone Receptor (PR) was markedly increased, as evident by immunohistochemical, FACS, and quantitative real-time-PCR (qPCR), analyses. Remarkably, these defects could be traced to a severe reduction in the number, self-renewal activity and differentiation potential of basal MaSC in Lbh null glands as quantified by FACS, mammosphere and in vitro differentiation assays. Mechanistically, we identified that LBH induces expression of epithelial stem cell
transcription factor ΔNp63, which acts downstream of LBH to promote a basal stem cell phenotype and mediates LBH-dependent repression of ERα.

Using functional tumor sphere assays in concert with RNAi mediated knockdown and stable overexpression of LBH in human breast cancer cell lines, we further demonstrate that LBH is required for breast CSC formation and maintenance and imparts invasive properties in these cells. Molecular investigation using qPCR and Western Blot analysis further showed that LBH, reminiscent of its role in normal MaSCs, promotes a CSC phenotype by inducing ΔNp63 and repressing the good-prognosis breast cancer marker ERα.

Lastly, by establishing a primary human mammary epithelial cell (HMEC) dedifferentiation model based on Tumor Growth Factor beta (TGFβ), we show that LBH may also implicated in the transformation of HMEC into CSCs through a morphogenetic process termed epithelial-mesenchymal transition (EMT). LBH most likely is induced by TGFβ at the transcriptional level, as we identified a conserved SMAD binding element (SBE) in the Lbh transcriptional start site that was TGFβ/SMAD3-responsive in transcriptional reporter assays.

Collectively, this thesis work identified the first essential role of LBH in promoting MaSC function and basal lineage specification in both normal and cancerous settings. Our findings provide important novel insight into the etiology of ‘triple-negative’ BC, and suggest that inhibition of LBH could be used as a method of ‘differentiation therapy’ to re-sensitize tumor cells to known endocrine therapies against ERα.
Acknowledgements

First, I would like to sincerely thank my mentor, Dr. Karoline Briegel for her unrelenting dedication to my scientific training. Her guidance, support and advice have been priceless in shaping me into the scientist I am today. I would also like to thank the entire Biochemistry and Molecular Biology Department at the University of Miami, Miller School of Medicine for the opportunity to pursue my doctoral studies and for their continued support in nurturing my academic and personal development throughout my time as an undergraduate and graduate student. I would like to thank my committee members Dr. Ralf Landgraf, Dr. Feng Gong, Dr. Dorraya El-Ashry and my external examiner Dr. David Robbins for their continued support and guidance throughout my graduate studies.

Lastly, my family and fiancé have been instrumental in helping me achieve my personal and professional goals and for that I will be forever grateful. Grayson, thank you for your unconditional love and never ending support throughout this graduate school experience.
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<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AER</td>
<td>Apical ectodermal ridge</td>
</tr>
<tr>
<td>ALDH</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>BCSC</td>
<td>Breast Cancer Stem Cell</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer Stem Cell</td>
</tr>
<tr>
<td>CHD</td>
<td>Congenital Heart Disease</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CK I/II</td>
<td>Casein Kinase 1 or II</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetraacetic Acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic Mobility Shift Assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to Mesenchymal Transition</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen Receptor alpha</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human Mammary Epithelial Cell</td>
</tr>
<tr>
<td>IDP</td>
<td>Intrinsically Disordered Protein</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>K</td>
<td>Keratin</td>
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<tr>
<td>KD</td>
<td>Knockdown</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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<tr>
<td>LBH</td>
<td>Limb-Bud and Heart</td>
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<tr>
<td>MaSC</td>
<td>Mammary Stem Cell</td>
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<tr>
<td>MEC</td>
<td>Mammary Epithelial Cell</td>
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<tr>
<td>MG</td>
<td>Mammary Gland</td>
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<tr>
<td>MiR</td>
<td>Micro RNA</td>
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<tr>
<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
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<tr>
<td>MRU</td>
<td>Mammary Repopulating Units</td>
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<tr>
<td>MW</td>
<td>Molecular Weight</td>
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<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>ORP</td>
<td>Open Reading Frame</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>Para Formaldehyde</td>
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<td>Progesterone Receptor</td>
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<tr>
<td>qPCR</td>
<td>Quantitative Real-Time PCR</td>
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<tr>
<td>RNAi</td>
<td>RNA Interference</td>
</tr>
<tr>
<td>SAN</td>
<td>Sinoatrial Node</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TBE</td>
<td>TCF/Lef Binding Element</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple Negative Breast Cancer</td>
</tr>
<tr>
<td>TEB</td>
<td>Terminal End Bud</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
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Chapter 1: Introduction
1.1 Mammary Gland Development

1.1.1 Mammary Gland Structure and Developmental Stages

The mammary gland (MG) is a complex secretory organ that possesses tremendous organizational plasticity and regenerative capacity throughout adult life. The MG comprises many different cell types including epithelial cells, which form the mammary ductal tree; adipocytes that make up the mammary fat pad; stromal cells and numerous types of immune cells. The mammary epithelium is a bi-layer consisting of an outer basal layer of contractile myoepithelial cells (Keratin5/6/14+; ER-) and an inner luminal layer of cells (Keratin8+; ER+/-) (Fig. 1.1) that can differentiate into secretory alveolar cells during pregnancy/lactation (Visvader 2009). This epithelial bi-layer is organized into a series of ductal and lobular structures that are nestled within the MG fat pad; the function of which is production and transportation of milk during pregnancy/lactation.

Figure 1.1: Cross-sections of a mammary gland (MG) duct (A) and terminal end bud (TEB) (B) depicting luminal epithelial cells in purple and basal-myoeipithelial cells in light red (adapted from Visvader 2009).
MG development takes place in three distinct phases: embryonic, pubertal and pregnancy/lactation (Fig. 1.3). Development of the mouse and human MG is similar despite human development being considerably more complex. This marked similarity allows the mouse to provide an excellent model system for studying the molecular underpinnings of MG development and thus will be the focus herein.

Approximately midway through gestation on embryonic day 10.5 (E10.5), two ridges of ectoderm referred to as milk lines (positive for Wnt10b) arise from the embryonic skin on the ventral surface of the embryo and give rise to 5 pairs of symmetrically positioned mammary placodes at E11.5 (Fig. 1.2A) (Veltmaat, Van Veelen et al. 2004, Watson and Khaled 2008). These placodes are not identically determined and have a distinct spatio-temporal appearance that can be perturbed by manipulation of key developmental transcription factors and pathways such as Wnt and Hedgehog (van Genderen, Okamura et al. 1994, Veltmaat, Mailleux et al. 2003, Hatsell and Cowin 2006). Loss of functional Wnt target gene $\text{Lef1}$ or key hedgehog signaling transcription factor GLI3 leads to improper placement, degeneration or complete lack of mammary placodes (van Genderen, Okamura et al. 1994, Veltmaat, Mailleux et al. 2003). Loss of GLI3 can be rescued through supplementation with FGF10; the normal gradient of which has been suggested to control mammary placode placement (Veltmaat, Relaix et al. 2006). Perturbation of developmental transcription factors TBX3 or GATA3 can also result in variable or complete loss of mammary placode
formation (Eblaghie, Song et al. 2004, Asselin-Labat, Sutherland et al. 2007) highlighting the complexity of this developmental process.

By E14.5, mammary epithelial buds have formed and invaginated into the dermis where they will elongate into mammary sprouts at E15.5 (Fig. 1.2C). Gene expression of multiple Wnt signaling molecules, \( Wnt, Fzd, Tcf, \) and \( Lef \) has been observed in dissected mammary buds and developing embryos at E12.5 and E15.5 and overexpression of the Wnt inhibitor Dickkopf 1 can completely abrogate bud formation (Chu, Hens et al. 2004). In female embryos, these epithelial sprouts invade the mammary fat pad and eventually form hollow lumen that connect to the skin where nipple formation will ultimately occur (Fig. 1.2D). Shortly thereafter the mammary sprouts begin to form rudimentary ductal trees that are arrested in development at E18.5 (Fig. 1.2D) until female mice enter puberty (Watson and Khaled 2008). In male
embryos normal degeneration of mammary buds occurs at E15.5 due to activation of androgen receptors.

At approximately 3-4 weeks of age female mice enter puberty and a new phase of “hormone-dependent” MG development. Production of ovarian-derived estrogen (E₂) marks the entrance into puberty. Estrogen is the major mitogenic stimulus during pubertal MG growth, mediating its effects through a cascade of events propagated by the action of Estrogen Receptor α (ERα), an intracellular steroid hormone receptor (Brisken and O'Malley 2010, Fridriksdottir, Petersen et al. 2011). During this phase, transient club-shaped structures called “terminal end buds” or TEBs that are located at the end of the rudimentary epithelial ducts are stimulated to proliferate. The TEBs consist of an inner multi-layer of body cells (luminal progenitors) and an outer layer of highly proliferative cap cells (basal-myoepithelial progenitors) (Fig. 1.1). The highly proliferative layer of cap cells is responsible for the growth of the mammary epithelium into the fat pad, a process also known as ductal elongation. This cell layer has been shown to be enriched in mammary stem cells, which actively self-renew and differentiate at this stage (Bai and Rohrschneider 2010). Regular cyclic secretion of progesterone occurs as mice approach sexual maturity between 8 weeks of age. At approximately 10-12 weeks of age the ductal tree reaches the edges of the mammary fat pad by means of continued ductal elongation and secondary branching/bifurcation (Fig. 1.3) (Silberstein 2001). Filling of the mammary fat pad is followed by regression of TEBs thus completing pubertal MG development (Watson and Khaled 2008).
The last phase of MG development takes place during pregnancy and lactation (Fig. 1.3). Pregnancy is characterized by the massive expansion of parity-induced stem cells (Asselin-Labat, Sutherland et al. 2007, Matulka, Triplett et al. 2007) resulting in tertiary branching of the ducts and formation of the milk-producing lobulo-alveolar compartment at the end of the ducts. Tertiary branching and the genesis of alveolar buds are controlled by the hormone progesterone, which mediates its downstream effects through Wnt4a (Bradbury, Edwards et al. 1995, Brisken, Park et al. 1998). Together progesterone and prolactin direct differentiation of luminal cells in the alveolar buds into secretory milk producing alveolar cells which begin secreting milk in late pregnancy/early lactation (Brisken, Park et al. 1998). Upon weaning of pups, the MG undergoes a massive reorganization to its pre-pregnancy state, which is mediated by a massive induction of programmed cell death or apoptosis, a process termed involution (Richert, Schwertfeger et al. 2000). Post-involution parous MGs return to a mostly non-parous state, a phenomenon that is repeated during everyone.

Figure 1.3: Post-natal mammary gland (MG) development. A schematic representation of MG development in the adult mouse, from pre-puberty (E18.5), puberty (3-8 weeks) to pregnancy, lactation and involution. Lymph node (LN); terminal end bud (TEB) (adapted from Watson and Khaled 2008).
pregnancy cycle and beautifully illustrates the plasticity and regenerative capacity of the MG (Fig. 1.3).

Such regenerative capacity is not held by all cells within the MG but is believed to be specific to a small subpopulation of multipotent mammary stem cells (MaSCs) and various unipotent progenitors within the mammary compartment. The complex cellular hierarchy giving rise to this MG will be discussed in greater detail in the next section.

1.1.2 Cellular Hierarchy and Mammary Stem Cells

![Figure 1.4: Mammary cellular hierarchy.](image)

The hierarchy of cell lineage specification within the MG is as of yet incompletely characterized, but it is believed that the two mature epithelial cell lineages (luminal, basal/myoepithelial) originate from a small population of mammary stem cells (MaSC) with basal-like (K5/6+; ∆Np63+; Axin2+; ER-)
characteristics (Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010). MaSC have the capacity to divide asymmetrically preserving their own lineage while also giving rise to bipotential progenitors from which unipotent luminal and myoepithelial progenitors arise (Visvader 2009, Van Keymeulen, Rocha et al. 2011). Cell fate studies in mice have demonstrated that only cells from the basal epithelial layer have the capacity to regenerate all mammary epithelial cell types and to form a complete MG tree in vivo, indicating that primitive MaSC reside in the basal/myoepithelial layer of the MG (Van Keymeulen, Rocha et al. 2011).

Evidence for the existence of multi-potent MaSCs has existed for more than 50 years. Groundbreaking work from Daniel and DeOme demonstrated that mammary epithelial tissue fragments transplanted into the cleared fat pads of recipient mice could repopulate the fat pad with a fully functional MG epithelial tree (Deome, Faulkin et al. 1959, Daniel, De Ome et al. 1968, Daniel, Young et al. 1971). These and future studies also demonstrated that repopulation of the fat pad was possible regardless of which area of the donor MG the mammary epithelial fragment was collected from and furthermore that these transplanted outgrowths could be serially transplanted up to 8 times before repopulation capacity was abrogated. The interpretation of these findings lead to the conclusion that there was a population of well dispersed cells within the MG that had the capability to repopulate a mammary fat pad when stimulated to do so and that these cells possessed a high degree of self-renewal that allowed them to perform this task repeatedly.
Since these pioneering studies, a great deal of effort has focused on identifying, characterizing and isolating putative MaSCs and their progenies. Electron and light-microscopy are techniques that have allowed the visualization of morphologically different cell types in situ. Using these methods, epithelial cells in the MG were characterized into four different types: primitive small light cells (SLC), undifferentiated large light cells (ULLC) differentiated large light cells (DLLC), and large dark cells (LDC) (Smith and Medina 1988, Chepko and Smith 1997). Due to their position, presence or absence of mitotically active chromosomes, organelle status and capacity to undergo symmetric or asymmetric division, these different cell types were judged to be more or less differentiated. At one end of the spectrum are the LDCs, which are differentiated myoepithelial and luminal cells and at the other end the SLCs, which have stem-like features and a basal or supra-basal position (Fig. 1.1) (Chepko and Smith 1997). SLCs were found to make up 3% of the total epithelial cell number and to be maintained throughout all normal MG developmental stages: virgin; pregnancy and involution (Chepko and Smith 1997). However, SLCs are depleted in serially transplanted mammary epithelial fragments concurrent with reduced self-renewal of these fragments over time, suggesting that these cells represent putative MaSC and are required for fat pad repopulation (Smith, Strickland et al. 2002).

Due to a lack of specific markers, it was not possible to directly isolate SLCs and confirm that the phenotypic characterization that has led to their categorization as MaSC is correct. Only recently, it has become possible to
enrich for putative MaSCs with long-term repopulating capacity termed “mammary repopulating units” or MRUs using cell surface markers (see Table 1.1 for a comprehensive list of markers) and fluorescence activated cell sorting (FACS) (Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006, Sleeman, Kendrick et al. 2007). Using combinations of antibodies specific for the cell surface markers CD24 (HSA) and CD29 (β1 integrin) primary mouse mammary cells can be sorted into luminal (CD29loCD24hi), basal myoepithelial/MRU/MaSC-enriched (CD29hiCD24+), and non-epithelial (CD29+CD24−) populations. Subsequent serial transplantation experiments using individual sorted cell populations from freshly dissociated virgin mammary tissue showed that only the basal epithelial cell fraction is enriched in MRUs, as evidenced by their ability to repopulate the cleared fat pads of host mice when injected in low numbers (Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006, Sleeman, Kendrick et al. 2007). In comparison, cells from the luminal epithelial population are extremely limited in their ability to generate MG outgrowths and completely unable to generate secondary outgrowths (Sleeman, Kendrick et al. 2007, Van Keymeulen, Rocha et al. 2011).

The capability to enrich for and transplant MRUs confirmed the existence of a unique set of stem/progenitor cells that had the capacity to give rise to all of the cells types within the MG but did not provide information on how these cells and more differentiated and/or committed progenitors function in MG homeostasis in vivo. Recent studies using genetic labeling techniques have elegantly delineated the physiological relevance of these MRUs or MaSCs and
more committed progenitors (Van Keymeulen, Rocha et al. 2011). Using a variety of inducible Cre recombinase expressing mouse strains to fluorescently label and track different cell lineages Van Keymeulen et al., 2011 demonstrated the existence of multi-potent Keratin 14/K14-positive basal MaSC, as well as unipotent K8+ luminal progenitor/stem-cells and long lived parity-induced luminal progenitors.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>CD24</td>
<td>HSA</td>
</tr>
<tr>
<td>CD29</td>
<td>β1 integrin</td>
</tr>
<tr>
<td>CD49f</td>
<td>α6 integrin</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Ly-6A/E</td>
</tr>
<tr>
<td>CD61</td>
<td>β3 integrin</td>
</tr>
<tr>
<td>Hoechst SP</td>
<td>Side population phenotype due to the Hoechst33342 efflux pump present on the plasma membrane. Activity conferred by the ABC transporter ABCG2.</td>
</tr>
<tr>
<td>CD49b</td>
<td>α2 integrin</td>
</tr>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>CD49f</td>
<td>α6 integrin</td>
</tr>
<tr>
<td>EpCAM</td>
<td>ESA, CD326</td>
</tr>
<tr>
<td>CD44</td>
<td>PGP1</td>
</tr>
<tr>
<td>CD90</td>
<td>THY1</td>
</tr>
<tr>
<td>CD133</td>
<td>Prominin 1</td>
</tr>
<tr>
<td>ALDH1</td>
<td>The ubiquitous ALDH family of enzymes catalyzes the oxidation of aliphatic and aromatic aldehydes to carboxylic acids. ALDH1 has a role in the conversion of retinol to retinoic acid.</td>
</tr>
<tr>
<td>MUC-1</td>
<td>CD227</td>
</tr>
<tr>
<td>CD24</td>
<td>HSA</td>
</tr>
<tr>
<td>CD10</td>
<td>CALLA</td>
</tr>
<tr>
<td>c-KIT</td>
<td>CD117</td>
</tr>
</tbody>
</table>

**Table 1.1:** Markers used to fractionate mammary epithelial cells into distinct cell populations in mouse and human mammary tissue (Visvader 2009).

In late embryogenesis (E17.5) and up until puberty K14 is expressed in both luminal and basal cells and only after the onset of puberty does K5/K14 expression become restricted to the basal lineage and excluded from luminal
cells, which instead express K8/18 (Van Keymeulen, Rocha et al. 2011). The distinct expression pattern of K14 during MG development is of relevance later in this thesis work as I will be using a K14 driven Cre recombinase to generate tissue specific Lbh null mice and based on studies presented here K14- Cre will direct gene ablation in all epithelial cells of the MG.

Interestingly, they further demonstrated that when a mixture of basal and luminal cells were transplanted into cleared fat pads at an equal ratio, the basal layer of the repopulated gland was only derived from the transplanted basal cells, and vice versa the luminal epithelial layer from transplanted luminal cells, as evidenced by fluorescent labeling and tracking of lineage (Van Keymeulen, Rocha et al. 2011). However, when basal and luminal cell populations were transplanted individually into cleared fat pads, only the basal/MRU/MaSC enriched population was capable of recapitulating a complete mammary epithelium, whereas the luminal population failed to do so (Van Keymeulen, Rocha et al. 2011). Thus, it appears that the ratio of basal-to-luminal cells is key and the basal/MRU/MaSC enriched population will only assume a multi-potent stem cell role contributing to both basal and luminal lineages when transplanted in excess to luminal cells, demonstrating the functional plasticity of these cells.

Additional studies using genetic in vivo fluorescent labeling have shown that activated, i.e. proliferating MaSC (p63+; CD24+CD29/49fhi) are enriched in the cap cells of TEBs during puberty and in the basal layer of alveolar buds during pregnancy (Bai and Rohrschneider 2010). These MaSC were isolated and shown to possess the functionally capacity to recapitulate an entire mammary
epithelial tree similar to the multi-potent MaSC identified by Van Keymeulen et al., 2011 (Bai and Rohrschneider 2010). Together these studies beautifully illustrate evidence for the existence of a multi-potent MaSC in the basal epithelial lineage that is functionally active during development and homeostasis throughout adult life. Furthermore, they provide important insight into the importance of cellular milieu and microenvironment for the physiological function and plasticity of mammary epithelial cells; a theme that has been the topic of much exploration in recent years. However, the molecular mechanisms governing the complex processes of lineage specification and MaSC homeostasis are still incompletely understood.

So far we have discussed studies which suggest a cellular hierarchical model of MaSC, which is predominantly unidirectional. However, mounting evidence suggests that there is a great deal of functional plasticity within the mammary epithelial tree and that modulation of extracellular stimuli can lead to the ‘de-differentiation’ of more luminal cells into basal MaSCs through a morphogenetic process termed the Epithelial to Mesenchymal Transition (EMT) (Mani, Guo et al. 2008, Morel, Lievre et al. 2008, Scheel, Eaton et al. 2011, Guo, Keckesova et al. 2012).

1.1.3 Key Pathways in Mammary Stem Cell Biology

Mammary gland development and homeostasis is a complex, highly orchestrated system directed by endocrine signals which regulate and act in
concert with a host of transcriptional regulators and developmental pathways to control proliferation and mediate cell lineage specification (Watson and Khaled 2008). Though the exact molecular mechanisms controlling MaSC biology, lineage specification and differentiation are incompletely understood, it is known that a collection of key developmental pathways: Wnt, TGF-β, Notch, and Hedgehog, are intimately involved and act upstream of key transcriptional regulators such as p63 (Li, Singh et al. 2008, Watson and Khaled 2008, Yalcin-Ozuysal, Fiche et al. 2010, Scheel, Eaton et al. 2011).

**Wingless Related Protein (Wnt) Pathway**

The Wnt signaling pathway is a conserved signaling network fundamental to embryonic development and normal stem cell function during adult tissue homeostasis (Logan and Nusse 2004, Reya and Clevers 2005, Clevers 2006, Klaus and Birchmeier 2008, Nusse 2008). Wnt ligands are lipid-modified secreted glycoproteins that can function through both, canonical and non-canonical pathways (Komiya and Habas 2008, van Amerongen, Mikels et al. 2008). The canonical Wnt pathway, in particular, is implicated in self-renewal of adult stem cells and this has been demonstrated in multiple organ systems including hematopoietic, epidermal, gut and mammary gland (Wright, Bowman et al. 2002, Reya, Duncan et al. 2003, Zeng and Nusse 2010).

Wnt ligands bind to a co-receptor complex consisting of members of the Frizzled (Fz) receptor family and their co-receptors: low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) (Schweizer and Varmus 2003). In the
canonical pathway, binding of Wnt ligand to Fz-LRP5/6 signals to halt phosphorylation of cytoplasmic β-catenin by Glycogen Synthase Kinase 3 beta (GSK3β) which is part of a degradation complex consisting of Adenomatous Polyposis Coli (APC) and Axin that normally sequesters β-catenin for degradation. Upon inhibition of phosphorylation and subsequent degradation, accumulation of β-catenin leads to nuclear localization and transcription of Wnt pathway targets thus mediating the self-renewal and stem-cell maintenance effects of the Wnt pathway (Logan and Nusse 2004). Although numerous target genes have been identified for the Wnt signaling pathway (refer to http://www.stanford.edu/group/nusselab/cgi-bin/wnt/ for a comprehensive review), few have been identified that can mediate the self-renewal effects of Wnt.

**Figure 1.5:** Schematic of the canonical Wnt signaling pathway. In the absence of Wnt ligands, cytoplasmic β-catenin is sequestered in a destruction complex (GSK3β/APC/Axin) where it is phosphorylated leading to its proteosomal degradation. In the presence of ligand, phosphorylation of β-catenin is inhibited and accumulation of protein leads to its translocation to the nucleus where it aids in transcriptional control.
Nusse and colleagues demonstrated in 2010 that Wnt ligands are extrinsic niche factors that directly act on MaSCs and serve as rate-limiting self-renewal signals controlling MaSC expansion and maintenance during MG development (Zeng and Nusse 2010). Studies in which components of the Wnt signaling pathway, such as Lrp5/6, and Pygo2, are inactivated through gene targeting in mice resulted in decreased numbers of TEBs, delayed ductal elongation, and a reduction in the MaSC-enriched basal epithelial pool accompanied by an impaired self-renewal potential of this population (Lindvall, Evans et al. 2006, Badders, Goel et al. 2009, Lindvall, Zylstra et al. 2009). Wnt1 was identified as the first mammary oncogene and inappropriate activation of WNT/β-catenin signaling in mouse mammary tumor virus (MMTV)-driven mouse models results in abnormal MaSC expansion and breast cancer, suggesting this pathway is intimately involved in MG biology and neoplasia (Nusse and Varmus 1982, Tsukamoto, Grosschedl et al. 1988, Li, Welm et al. 2003, Liu, McDermott et al. 2004, Teuliere, Faraldo et al. 2005, Teissedre, Pinderhughes et al. 2009). Despite the low frequency of Wnt pathway mutations in breast cancer (Brennan and Brown 2004), β-catenin has been found to be stabilized in ~50% of breast cancers and correlates with a poor outcome (Lin, Xia et al. 2000). Moreover, our group and others have shown that Wnt pathway hyperactivation is associated with poor prognosis basal-like, triple negative breast cancers (Khramtsov, Khramtsova et al. 2010, Rieger, Sims et al. 2010), which are known to be highly aggressive and prone to metastasis (Lakhani, Reis-Filho et al. 2005, Fulford, Easton et al. 2006, Gluz, Liedtke et al. 2009). It has been shown that this type of
cancer is enriched in cells that possess stem-like properties: so called ‘breast cancer stem cells’ (BCSCs; discussed further in Section 1.2.3), the genesis and maintenance of which have been linked to the combined activities of Wnt and Transforming Growth Factor beta (TGFβ) signaling (Scheel and Weinberg 2011).

**Transforming Growth Factor Beta (TGFβ) Signaling Pathway**

The TGFβ pathway is multifunctional signaling network, playing diverse roles in cellular proliferation, differentiation, migration, adhesion and also in EMT, a process crucial to embryonic development, wound healing, and malignant tumor progression (Moses and Barcellos-Hoff 2011). EMT is characterized by the loss of epithelial cell-to-cell contacts with a decrease in epithelial adhesion molecule E-cadherin, loss of epithelial cell polarity, a major reorganization of the cytoskeleton, an increase in mesenchymal marker expression, and gain of a fibroblastic, motile cell phenotype (Thiery, Chua et al. 2010).

There are three soluble TGFβ ligands (TGFβ1-3) that are secreted in a latent form that is proteolytically cleaved to an active form and three TGFβ receptors (TGFβRI-III). TGFβ ligands 1 and 3 can bind directly to cell surface receptor TGFβRII, a serine threonine kinase, while TGFβ2 requires the presence of TGFβIII to efficiently bind TGFβRII. Upon binding, TGFβRI is recruited and activated, leading to activation of the canonical TGFβ pathway and phosphorylation of intracellular SMAD proteins (Fig. 1.6). Upon phosphorylation, SMAD 2 or 3 form a complex with SMAD 4 that then translocates to the nucleus,
where it aids in activation or repression of target genes (Fig. 1.6) (Moses and Barcellos-Hoff 2011).

**Figure 1.6:** Canonical Transforming Growth Factor beta (TGF-β) signaling pathway. TGF-β ligands bind to TGFβ receptors at the cell surface leading to phosphorylation of Smad2/3 which then form a complex with Smad4 that translocates to the nucleus to aid in transcriptional control. (Hui and Lindeman 2003)

In the MG, TGFβ is normally expressed in stromal cells and functions to locally inhibit epithelial cell growth during branching morphogenesis, TEB regression after puberty, and clearing of mammary tissue during involution (Moses and Barcellos-Hoff 2011). TGFβ is also a major inducer of EMT, and published data suggests that during TGFβ mediated EMT, cells undergo a dedifferentiation process resulting in the acquisition of increased self-renewal capacity, an enhanced ability to form mammospheres *in vitro* and the acquisition of a more ‘stem-like’ cell surface antigen profile (Mani, Guo et al. 2008, Morel, Lievre et al. 2008). Additional evidence for a physiological role of TGFβ in MaSC biology was provided in a study by Weinberg and colleagues showing that primary MaSC enriched mammary epithelial cells display higher levels of nuclear SMAD2 than do their more differentiated counterparts (Scheel, Eaton et al.
Furthermore, inhibition of autocrine TGFβ signaling lead to reduced motility, reduced growth in suspension mammosphere culture and reduced levels of MaSC markers SLUG and p63 (Scheel, Eaton et al. 2011). Conversely, treatment with TGFβ increased the mammosphere forming ability of mammary epithelial cells, an effect that was further augmented upon pre-treatment with Wnt ligands, highlighting the synergistic capacity of these two developmental pathways in promoting a MaSC state (Scheel, Eaton et al. 2011).

Despite its normal growth inhibitory and tumor suppressor role, aberrant TGFβ signaling has been found to be re-activated and expressed at chronically high levels in invasive tumors, and in inflamed or fibrotic tissues (Zavadil and Bottinger 2005, Thiery, Chua et al. 2010). During pathological situations such as fibrosis and inflammation and during carcinogenesis aberrant TGFβ signaling may promote tumor progression and invasion through EMT (Thiery, Chua et al. 2010). EMT of transformed epithelial cells can lead to de-differentiation and the acquisition of a cancer stem cell (CSC) phenotype thus imparting greater self-renewal and motile/invasive properties allowing such cells to invade and potentially form metastasis (Frixen, Behrens et al. 1991, Yang, Mani et al. 2004, Mani, Guo et al. 2008, Morel, Lievre et al. 2008, Sabbah, Emami et al. 2008, Taube, Herschkowitz et al. 2010).
The cell intrinsic control of MaSC regulation is much less well understood. While numerous transcription factors have been shown to be required for luminal epithelial cell specification and differentiation, such as GATA3, ELF5, C/EBPβ, and STAT6 (Kouros-Mehr, Slorach et al. 2006, Choi, Chakrabarti et al. 2009, LaMarca, Visbal et al. 2010, Chakrabarti, Wei et al. 2012, Oliver, Khaled et al. 2012), only very few transcription factors controlling basal/MaSC fate have been identified. The most relevant for this thesis is p63, a p53 and p73 homolog that plays important roles in epithelial stasis and stem cell self-renewal (Mills, Zheng et al. 1999, Yang, Schweitzer et al. 1999, Senoo, Pinto et al. 2007). TP63 deficient mice have severely impaired development of skin and a complete lack of MG development, a phenotype attributed to non-regenerative differentiation (Mills, Zheng et al. 1999, Parsa, Yang et al. 1999, Yang, Schweitzer et al. 1999, Signoretti, Waltregny et al. 2000). Two p63 isoforms are produced from differential promoter usage: ΔNp63 and TAp63 (Yang, Schweitzer et al. 1999). ΔNp63 is expressed in the stem cell niche of many tissues including the breast and numerous functional studies provide evidence that this isoform is responsible for self-renewal of stem cells. In contrast, TAp63 is expressed in more differentiated luminal cells of the MG (Yang, Schweitzer et al. 1999, Nylander, Vojtesek et al. 2002, Li, Li et al. 2006, Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010). Studies in breast epithelial cells indicate that experimental down-modulation of ΔNp63 in basal/MaSC enriched cell populations leads to differentiation of these cells down a luminal lineage and conversely ectopic
expression of ΔNp63 in luminal cells leads to more basal/MaSC characteristics (Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010).

Studies by Badders have hinted that p63 may be regulated by Wnt as ΔNp63 is reduced in Lrp5\(^{-/-}\) MECs and similar to Lbh is overexpressed in tumors from MMTV-Wnt1 transgenic mice (Badders, Goel et al. 2009, Rieger, Sims et al. 2010). TCF/LEF binding elements have been identified in the promoter sequences for p63 indicating that WNT signaling could potentially promote the observed repression of TAp63 and expression of ΔNp63 required for maintenance of the a MaSC phenotype (Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010). ΔNp63 is also involved in direct positive feed-back regulation of two key MaSC maintenance and self-renewal pathways: WNT and TGFβ (Patturajan, Nomoto et al. 2002, Lindsay, McDade et al. 2011, Balboni, Hutchinson et al. 2013). ΔNp63 positively regulates the Wnt/β-catenin pathway and nuclear accumulation of β-catenin directly through inhibition of GSK3β-mediated phosphorylation and degradation of β-catenin (Patturajan, Nomoto et al. 2002) as well as the TGFβ pathway through transcriptional activation of TGFβ1 ligand and other superfamily components (Lindsay, McDade et al. 2011, Balboni, Hutchinson et al. 2013). In contrast to the MaSC maintenance functions of p63 in the context of Wnt and TGFβ signaling, Hedgehog and Notch signaling have both been shown to play a key role in mediating a p63 switch by down-modulating ΔNp63 and subsequently upregulating TAp63 during luminal progenitor cell elaboration (Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010).
1.2 Breast Cancer

1.2.1 Breast Cancer Overview

Breast cancer is the second leading cause of cancer related death in women with 1 in every 8 women being diagnosed with breast cancer in their lifetime (American Cancer Society; www.cancer.org), though certain genetically inherited mutations in BRCA genes increase this risk substantially (Maxwell, Benitez et al. 2011). Histological classification along with tumor grade: a measure of the degree of differentiation and proliferation, provide prognostic value in determining patient outcome (Elston and Ellis 1991, Ellis, Galea et al. 1992). Clinical pathology employs immunohistochemical techniques to evaluate expression of three cell surface receptors: Estrogen Receptor (ER), Progesterone Receptor (PR) and human epidermal growth factor receptor (HER2/ERBB2/NEU) to aid in proper formulation of clinical treatment plans (Harvey, Clark et al. 1999). Both ER and PR positivity are indicative of response to targeted therapies such as Tamoxifen (Fisher, Redmond et al. 1983, Stierer, Rosen et al. 1993), while HER2 positive tumors may be treated with HER2 targeted therapies such as Trastuzumab (Piccart-Gebhart, Procter et al. 2005). ER/PR positive, HER2 negative cancers are most common and have better survival outcomes as compared to ER/PR/HER2 negative or “triple negative” cancers, which have the poorest prognosis (Onitilo, Engel et al. 2009). In an effort to enhance prognosis and therapeutically efficacious treatment plans
breast cancers have also been molecularly profiled and classified into groups with distinct signatures that will be discussed in greater detail in the next section (Section 1.2.2).

1.2.2 Breast Cancer Molecular Subtypes

Microarray profiling of primary breast tumors has identified 5 major ‘intrinsic’ molecular subtypes with important clinical relevance: Luminal A, Luminal B, Normal-like, HER2+, and Basal tumors which also have additional sub-categories including claudin-low and metaplastic tumors (Perou, Sorlie et al. 2000, Sorlie, Perou et al. 2001, Sorlie, Tibshirani et al. 2003, Hu, Fan et al. 2006, Herschkowitz, Simin et al. 2007, Hennessy, Gonzalez-Angulo et al. 2009). Prognosis and clinical course are inherently different between each of the molecular tumor subtypes as outlined in Table 1.2. Luminal A subtype cancers have the best prognosis as they are hormone receptor positive (ER/PR+), well differentiated and can be treated with Tamoxifen (Sims, Howell et al. 2007). Many Luminal B cancers lack expression of ER, are less differentiated and have a poorer prognosis than Luminal A cancers, however those that are ER+ can still be treated with endocrine therapies (Sorlie, Perou et al. 2001, Sorlie, Tibshirani et al. 2003, Sims, Howell et al. 2007). Normal-like breast cancers are an anomalous group that are typically ER- but are thought to be a mixed subtype due to contamination of normal breast tissue during tissue collection (Parker, Mullins et al. 2009). HER2 positive tumors are mixed in hormone receptor
positivity and can be targeted with therapies like Trastuzumab (HER2 antibody) leading to an overall better prognosis as compared to HER2- tumors (Dawood, Broglio et al. 2010).

Finally, basal subtype tumors encompass ER/PR/HER2-negative or ‘triple negative’ breast cancers (TNBCs), which are highly aggressive and have the worst clinical outcome (Carey, Perou et al. 2006, Millikan, Newman et al. 2008). Despite representing only 15-20% of breast cancer cases, basal breast cancer accounts for a disproportionately high percentage of breast cancer related deaths (Gluz, Liedtke et al. 2009). Basal subtype tumors have a high mitotic index, are highly invasive and have a higher incidence of metastasis than other subtypes (Lakhani, Reis-Filho et al. 2005, Fulford, Easton et al. 2006, Gluz, Liedtke et al. 2009). As previously mentioned, there are genetically inherited mutations in BRCA1/2 genes that result in a 20-30 fold greater risk of developing breast cancer and these tumors usually resemble basal subtype (Lakhani, Reis-Filho et al. 2005).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Prognosis</th>
<th>Clinical Markers</th>
<th>Additional Markers</th>
</tr>
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<tr>
<td>Normal-like</td>
<td>Mixed</td>
<td>ER+/-, PR+/-, HER2-</td>
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</tr>
<tr>
<td>Luminal A</td>
<td>Good</td>
<td>ER+, PR+, HER2-</td>
<td>CK8/18+, GATA3+</td>
</tr>
<tr>
<td>Luminal B</td>
<td>Poor</td>
<td>ER+/-, PR+/-, HER2-</td>
<td></td>
</tr>
<tr>
<td>ERBB2</td>
<td>Good</td>
<td>ER+/-, PR+/-, HER2+</td>
<td>GRB7</td>
</tr>
<tr>
<td>Basal</td>
<td>Worst</td>
<td>ER-, PR-, HER2-</td>
<td>CK5/6+, CK17+, CK14+, c-KIT+, high Ki67, EGFR+, P03+, P-cadherin+</td>
</tr>
</tbody>
</table>

Table 1.2: Breast cancer molecular subtypes with associated prognosis and clinical marker expression (Adapted from Matos et al., 2005; Ribeiro-Silva et al., 2005; Sims et al., 2007; Weigelt et al., 2010)
Regardless of etiology, there are no targeted therapies available to those patients diagnosed with basal subtype, TNBC (Gluz, Liedtke et al. 2009). Chemotherapy is the first line treatment option for these types of tumors; however recurrence commonly occurs within 3 years of treatment (Gluz, Liedtke et al. 2009, Voduc, Cheang et al. 2010), and it has been hypothesized that this is due to these tumors harboring a high percentage of treatment resistant ‘breast cancer stem cells’ (BCSCs; discussed in greater detail in Section 1.2.3).

1.2.3 Cell of Origin and the Breast Cancer Stem Cell (BCSC)

One proposed hypothesis for the genesis of different breast tumor subtypes is that they are each derived from a different ‘cell of origin’ providing them with inherently different properties (Fig. 1.9). For instance, it has been proposed that more differentiated tumor subtypes like Luminal A/B might be derived from a more differentiated luminal cell of origin while less differentiated subtypes like basal might be derived from basal stem cell/progenitor cells. Comparative analysis of gene expression profiles from freshly isolated populations of primary human mammary epithelial cells (MECs) and different breast cancer subtypes supports this hypothesis (Lim, Vaillant et al. 2009). These studies confirmed that Luminal A/B subtypes have a molecular signature that is similar to differentiated luminal epithelial cells (Lim, Vaillant et al. 2009). Furthermore, Claudin-low and metaplastic tumors, which are subtypes of basal TNBCs, displayed a gene signature comparable to the MaSC enriched, normal
basal MEC population (Lim, Vaillant et al. 2009). Surprisingly however, many basal subtype and BRCA1 mutation carrying tumors demonstrated a striking resemblance to an ER- luminal progenitor signature, indicating that luminal progenitors may undergo oncogenic dedifferentiation events in the genesis of basal subtype cancers (Lim, Vaillant et al. 2009, Molyneux, Geyer et al. 2010). Together these findings provide information about the cellular lineage from which different subtypes of cancer may arise within the breast (Lim, Vaillant et al. 2009, Visvader 2011); however it does not directly explain the high degree of heterogeneity within any one tumor.

Tumor subtypes that are derived from stem or progenitor cells likely have the propensity to differentiate into multiple cell types resulting in heterogeneous tumors while still maintaining the molecular signature of the cells from which they arose in a model termed the ‘Cell of Origin Model’ (See Fig. 1.7B). Alternatively the ‘Genetic Mutation Model’ (Fig. 1.7a) postulates that different subtypes of cancer arise from a common cell type with the tumor phenotype being determined by a collection of genetic and/or epigenetic events that are propagated through clonal selection (Visvader 2011). Most likely, a tumor phenotype is determined by a combination of both cellular origin and acquired genetic instability and clonal selection.
The first evidence for BCSCs came from seminal studies by Al-Hajj et al in 2003, who identified a small population of cells within breast tumors that harbor tumor-initiating capacity, and can additionally give rise to more differentiated progeny (usually less competent to form new tumors). These cells were termed BCSCs, because they share functional: increased self-renewal, and phenotypic: similar cell surface antigens, with normal MaSCs (Al-Hajj, Wicha et al. 2003). BCSCs are highly malignant and chemotherapy resistant and are thus thought to be a potential driver of tumor recurrence (Jones, Matsui et al. 2004, Creighton, Chang et al. 2010). Additionally, BCSCs display a mesenchymal cell morphology, an EMT gene expression signature, and are highly invasive with increased metastatic potential (Sheridan, Kishimoto et al. 2006, Shipitsin, Campbell et al. 2007, Fillmore and Kuperwasser 2008).

Experimentally BCSCs can be isolated from dissociated tumors by labeling and sorting with cell surface antigens CD24 (HSA) and CD44 (PGP1).
and similar to normal human MaSC, have a \(\text{CD}44^{\text{hi}}\text{CD}24^{\text{low/-}}\) antigenic phenotype (Al-Hajj, Wicha et al. 2003). \textit{In vivo}, as few as 200 isolated BCSCs can be injected into mice to give rise to tumors while thousands of more differentiated (\(\text{CD}44^{\text{low/-}}\text{CD}24^{\text{hi}}\)) tumor cells must be injected to initiate tumor growth (Al-Hajj, Wicha et al. 2003). Moreover, BCSCs can be passaged multiple times \textit{in vivo} and will give rise to tumors with the same phenotypic heterogeneity found in the primary tumor from which they were isolated (Al-Hajj, Wicha et al. 2003). As compared to other subtypes, aggressive basal subtype tumors are enriched in \(\text{CD}44^{\text{high}}\text{CD}24^{\text{low/-}}\) BCSCs (Honeth, Bendahl et al. 2008), especially claudin-low and metaplastic basal tumors, which are also are enriched in an EMT gene signature characteristic for BCSCs (Fillmore and Kuperwasser 2008, Taube, Herschkowitz et al. 2010). The tumorigenic, metastatic and therapy-resistant nature of BCSCs makes them a therapeutically advantageous target for currently uncurable metastatic cancers and highlights the need for new therapies targeted towards this population of cells.

1.3 Limb-Bud and Heart (LBH)

1.3.1 Identification and Molecular Characterization

Limb-bud and heart (LBH) was initially discovered in a yeast two hybrid screen while searching for interacting partners for the mouse homeodomain transcription factor Engrailed 1 (En1) (Briegel and Joyner 2001). A Xenopus Lbh orthologue, Xlcl2, was cloned prior to this as a maternal RNA in oocytes with
unknown function that becomes polyadenylated, and presumably translated into protein, upon fertilization (Paris, Osborne et al. 1988, Paris and Richter 1990). Further characterization of \( Lbh \) was pursued after it was found to exhibit a striking and unique expression pattern during mouse embryonic development (Briegel and Joyner 2001). \( Lbh \) maps to mouse chromosome 17 band E2 and maps to human chromosome 2p23 (Briegel, Baldwin et al. 2005). The \( Lbh \) locus encompasses a region of approximately 30 kb, and contains 3 exons encoding an mRNA of 3,086 nucleotides (murine; 2,933 bp in humans; Fig. 1.8) and a protein of 105 amino acids (Briegel and Joyner 2001). The \( Lbh \) transcript encompasses coding exon 1 (amino acids 1-8), exon 2 (amino acids 9-43), and exon 3 (amino acids 44-105), which before splicing are separated by 2.6 kb and 18 kb introns respectively. The 5’ and 3’ Untranslated Regions (UTRs) are 248 bp and 2,501 bp in the mouse and 208 and 2,410 in the human \( Lbh \) transcripts. Only one bona fide protein coding transcript has been identified by Northern blot analysis using various cDNA probes (Briegel and Joyner 2001, Ai, Wang et al. 2008).

**Figure 1.8:** Schematic of the LBH protein including domains: N-terminus hydrophobic stretch (green); nuclear localization signal (NLS, blue); C-terminus acidic domain (red) and putative phosphorylation sites as determined by Scansite.
*Lbh* encodes a small intrinsically disordered protein containing an amino terminal hydrophobic region, a putative nuclear localization signal (NLS), and a glutamate-rich putative transcriptional activation domain, but no direct DNA-binding domain (Fig. 1.8) (Briegel and Joyner 2001, Al-Ali, Rieger et al. 2010). Biophysical and biochemical analysis revealed that LBH has a disordered protein structure indicating it may belong to a family of intrinsically disordered proteins (IDPs) allowing for multiple interactions with diverse binding partners (Al-Ali, Rieger et al. 2010). Further supporting the notion that LBH is intrinsically unstructured is the experimental documentation that LBH consistently migrates at 17kDa in SDS-PAGE analysis despite its predicted molecular weight of 12.3kDa (Briegel and Joyner 2001, Al-Ali, Rieger et al. 2010, Rieger, Sims et al. 2010). Transcription factors commonly possess activation domains that contain acidic moieties in the carboxyl terminus as does LBH (Ptashne and Gann 1997), which contains a predicted alpha helix at position 85-101, indicative of a protein-protein interaction site (Briegel and Joyner 2001, Al-Ali, Rieger et al. 2010).

*In silico* analysis revealed numerous putative phosphorylation sites at positions at S34, S49, S63, T69, and T98 (Ai, Wang et al. 2008, Rieger, Sims et al. 2010) that are conserved among the majority of vertebrate species (Yaffe 2003) (http://scansite.mit.edu). Both T69 and T98 are conserved in mammals and are predicted to be phosphorylated by Casein Kinase 2 (CK2) and Protein Kinase C (PKC) respectively, while S34 is predicted to be phosphorylated by MAPK and/or CDK1 (Ai, Wang et al. 2008, Rieger 2010) (Figure 1.8; ELM, MetPHosK1.0). Both S63 and T69 are located near the putative NLS, suggestive
of a role in regulating cellular localization of LBH, as phosphorylation of amino acids near NLS have been shown to play an important role in nuclear localization of transcription factors (Brunet, Park et al. 2001, Macian, Lopez-Rodriguez et al. 2001, Holmberg, Tran et al. 2002). Experimental phosphatase and phosphatase inhibition treatment of endogenous LBH protein extracts revealed the presence of phosphorylated species (Rieger 2010), however these results have not yet been confirmed by mass spectrometry and warrant further investigation.

### 1.3.2 Cellular Localization and Transcriptional Activity

In mammalian cell lines, exogenously expressed epitope-tagged LBH, as well as endogenous LBH protein can be detected by an LBH-specific antibody and are predominantly nuclear (Briegel and Joyner 2001, Ai, Wang et al. 2008, Rieger, Sims et al. 2010). However, during mouse embryonic development in vivo, a tissue specific pattern of varying degrees of nuclear and cytoplasmic localization is observed (Rieger 2010). For instance LBH protein was found to be both nuclear and cytoplasmic in the heart, eye, and skin ectoderm at E10.5 and E14.5, while it was completely excluded from the nucleus and 100% cytoplasmic in differentiated post-mitotic neural cells in the trigeminal and dorsal root ganglia at these stages (Rieger 2010). Based on these results, it is plausible that the cellular localization of LBH is tightly regulated and that it may have different functions in the cytoplasm and nucleus and/or that its localization may determine its activation.
The transcriptional activity of LBH was first demonstrated using a Gal4 reporter assay, whereby a C-terminal fusion of the entire Lbh Open Reading Frame (ORF) was fused to the Gal4 DNA binding domain (Briegel and Joyner 2001). The Lbh-Gal4 fusion stimulated luciferase reporter activity 10-20 fold as compared to basal luciferase expression, indicating a role for LBH as a transcriptional activator (Briegel and Joyner 2001). This finding was further substantiated by studies indicating that transient transfection of human LBH in HeLa cells resulted in stimulation of SRF and AP-1 luciferase constructs (Ai, Wang et al. 2008). In addition to these initial studies implicating LBH in transcriptional activation, the majority of subsequent in vivo studies have highlighted LBH as a transcriptional co-repressor (Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009). Transgenic mis-expression of LBH in the developing myocardium of mice using a Carp-Lbh transgene, as well as heterologous transient co-expression reporter assays led to a functional repression of key cardiac transcription factors Nkx2.5 and Tbx5 (Briegel, Baldwin et al. 2005). Moreover, retroviral overexpression of Lbh in developing chick limbs resulted in repression of bone transcription factor Runx2 and proangiogenic factor Vegf at the transcriptional level (Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009). In vivo data presented in later chapters of this thesis suggest that LBH can act both as a transcriptional co-activator and co-repressor (Chapters 4-5).
1.3.3 Expression and functional role during embryonic development

The expression pattern of \( Lbh \) during embryonic development has been comprehensively evaluated using RNA \( in situ \) hybridization in developing mouse embryos (Briegel and Joyner 2001). During mouse embryonic development, LBH is expressed after gastrulation with a unique spatio-temporal gene expression pattern in the embryonic limb buds, heart, gut, kidney, gonads, peripheral nervous system, brain, and bones (Briegel and Joyner 2001, Briegel, Baldwin et al. 2005, Hoogaars, Engel et al. 2007, Conen, Nishimori et al. 2009). Expression of \( Lbh \) is first detected at E7.5 shortly after gastrulation in the primitive cardiac mesoderm and gut endoderm (Briegel and Joyner 2001). At E9.5 \( Lbh \) is strongly expressed with a right-left asymmetric expression pattern in the ventricle of the heart, as well as in the apical ectodermal ridge (AER) of the developing limb-buds and ventral limb ectoderm (Fig. 1.9) (Briegel and Joyner 2001). Interestingly, in chick embryos \( Lbh \) is specifically expressed in the dorsal Hensen’s node (Alev, Wu et al.), which like the AER acts as an organizer in tissue formation and is a rich source of morphogenetic signals (Wnt, Bmps etc.).

This spatio-temporal pattern of \( Lbh \) during organogenesis suggested that \( Lbh \) might be regulated by morphogenic pathways that control pattern formation and cell specification (Briegel and Joyner 2001). This notion was confirmed by studies conducted by our lab where we demonstrated that \( Lbh \) is a direct target gene of the morphogenetic WNT signaling pathway, being regulated by a critical balance between canonical and non-canonical Wnt signaling (Rieger, Sims et al. 2010) (Wnt regulation will be further discussed in section 1.3.6.). Additionally, my
preliminary data indicates that \textit{Lbh} expression is also positively controlled by TGFβ signaling, which plays a major role in induction of EMT (Chapter 5), a process crucial for development, tissue homeostasis, and tumor progression, as previously mentioned (Section 1.1.3).

Later in development \textit{Lbh} is expressed in numerous epithelial compartments such as the oral epithelium and otic vesicles and is also expressed in various components of the nervous system including the dorsal root and trigeminal ganglia (Briegel and Joyner 2001). By E17.5 \textit{Lbh} expression is also found in the sino-atrial node of the atria (SAN), which functions as the pace maker of the heart, and this expression was found to be downstream of Tbx3, as \textit{Lbh} is lost in the SAN of \textit{Tbx3}-deficient mice (Hoogaars, Engel et al. 2007). The \textit{Xenopus} \textit{Lbh} ortholog \textit{Xicl2} is also specifically expressed during heart development, suggesting that \textit{Lbh} function in this organ may be conserved between species (Gawantka, Pollet et al. 1998).

As mentioned, \textit{in vivo} LBH gain-of-function studies have been conducted in mouse heart and chick limb development (Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009). In both instances mis-expression of \textit{Lbh} resulted in prolonged proliferation as well as delayed differentiation of progenitor cells, suggestive of both a pro-mitogenic role as well as a possible stem cell/ progenitor

\textbf{Figure 1.9:} At E9.5 \textit{Lbh} mRNA expression is predominantly restricted to the heart and limb buds as shown by whole mount \textit{in situ} hybridization (left panel). Cross sections of the heart (a) and limb buds (b) demonstrate a right-left and dorso-ventral asymmetry, respectively (Briegel and Joyner, 2001, modified).
maintenance role for Lbh (Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009). In the heart, *Lbh* expression, which normally is restricted to the right ventricle, outflow tract, atrial ventricular canal and SAN (Briegel and Joyner 2001, Hoogaars, Engel et al. 2007) (Fig. 1.9a), was uniformly driven by a heart specific promoter *Cardiac Ankyrin Repeat Protein (Carp)* in the developing myocardium, resulting in a spectrum of outflow tract, septation, and ventricular growth defects (Briegel, Baldwin et al. 2005). Improper cardiomyocyte differentiation likely accounts for the cardiac defects in this mouse model and is a result of downregulation of *Nppa/ANF* through functional repression of key cardiac transcription factors Nkx2.5 and Tbx5 by Lbh (Briegel, Baldwin et al. 2005). In chick limbs ectopic expression of *Lbh* resulted in downregulation of bone transcription factor *Runx2* and proangiogenic growth factor *Vegf* mRNA expression causing a delay in bone formation and vasculogenesis (Conen, Nishimori et al. 2009).

Interestingly, *Carp-Lbh* mice phenocopied congenital heart disease of human patients with partial trisomy 2p syndrome, which is due to triplication of chromosomal region 2p23, where *LBH* is located (Briegel, Baldwin et al. 2005). Partial trisomy 2p syndrome is a rare human autosomal disorder characterized by multiple congenital anomalies including cardiovascular, skeletal and postaxial limb malformations (Lurie, Ilyina et al. 1995). The aforementioned defects observed upon ectopic overexpression of LBH in chick embryonic limbs (Conen, Nishimori et al. 2009) might also suggest that aberrant LBH gain contributes to
skeletal defects in these patients. Together these in vivo findings suggest that LBH acts as a transcription co-repressor with multiple roles in organogenesis.

1.3.4 Expression in adult tissues and the Mammary Gland

Expression of LBH in an array of adult tissues such as the heart, spleen, kidney and brain was identified using Northern blot analysis (Briegel and Joyner 2001, Ai, Wang et al. 2008). Global microarray analysis of human and mouse tissues also demonstrated high levels of expression in heart, lung, thymus, lymph node and several lineages of the hematopoietic system (Su, Wiltshire et al. 2004). Most relevant to this thesis, however, is the expression pattern of Lbh in the developing MG, which was first described by Rieger et al in 2010. In situ RNA hybridization analysis indicates that expression of Lbh in 7-week-old virgin female mouse MGs is restricted to basal-myoepithelial, TEBs, and stromal cells; whereas it is mostly

![Figure 1.10: Lbh expression during mouse mammary gland (MG) development. (A) RNA in situ hybridization analysis of sagittal cryosections of virgin, mid-pregnancy, lactating and early involution MGs (40x). Lbh is expressed in basal-myoepithelial, TEBs and stromal cells in virgin MGs, as well as in the lobuloalveolar units during pregnancy and involution. (B) WB analysis depicting Lbh protein levels during normal MG development at the same stages as in (A) (Rieger 2010 modified).]
excluded from the ductal luminal cells (Fig. 1.10) (Rieger, Sims et al. 2010). During pregnancy and early involution $Lbh$ mRNA is primarily localized to the expanding proliferative luminal lobuloaveolar compartments (Fig. 1.10) (Rieger, Sims et al. 2010). However, virtually no $Lbh$ mRNA and protein expression is detected in the lactating MG, suggesting that $Lbh$ is excluded from differentiated milk producing secretory mammary epithelial cells (Fig. 1.10) (Rieger, Sims et al. 2010). Further characterization and validation of micro-array data (Lim, Wu et al. 2010) corroborating the expression pattern of $Lbh$ in the MaSC compartment will be explored in Chapter 4.

1.3.5 Regulation of $Lbh$ by the Wnt/β-catenin Pathway

The unique expression pattern of $Lbh$ during embryonic development hinted that it might be regulated by pathways involved in directing morphogenesis and differentiation (Briegel and Joyner 2001). *In silico* analysis revealed four conserved consensus TCF/LEF binding motifs (TEBs) (5’-CTTTG(A/T)(A/T)-3’) (van de Wetering, Oosterwegel et al. 1991) in the $Lbh$ gene locus (Rieger, Sims et al. 2010). Two TBEs are localized in an enhancer region -6245 bp and -6195 bp upstream of the transcriptional start site (T1 and T2), and another two in an enhancer contained within the first intronic region of the (+1558 bp; +2145 bp) $Lbh$ locus (T3 and T4) (Rieger, Sims et al. 2010). Electrophorectic mobility shift analysis (EMSA) demonstrated that recombinant TCF4 protein bound specifically and with high affinity to all $Lbh$-specific TBEs (Rieger, Sims et
Luciferase promoter constructs containing wild-type and individual mutations in each TBE or combinations thereof determined the \textit{in vivo} activity of these sites when co-transfected with constitutively active $\beta$-catenin into HC11 mouse mammary epithelial cells (Rieger, Sims et al. 2010).

Furthermore, endogenous \textit{Lbh} expression has been shown to be induced in mammalian 293T and HC11 cells upon treatment with canonical Wnt3a ligand whereas treatment with non-canonical Wnt7a abrogated Wnt3a-induced expression of \textit{Lbh} (Rieger, Sims et al. 2010). Chromatin immunoprecipitation (ChIP) analysis further confirmed the \textit{in vivo} binding of $\beta$-catenin to the TBEs in the \textit{Lbh} locus upon treatment of Wnt3a in HC11 cells (Fig. 1.11) (Rieger, Sims et al. 2010). An antagonistic regulation of Wnt-beta-catenin induced \textit{Lbh} expression by the non-canonical Wnt signaling was also observed \textit{in vivo} during limb development as \textit{Lbh} was mis-expressed in the distal dorsal limb ectoderm in Wnt7a$^{/-}$ mice, where it is normally absent due to expression of Wnt7a as is seen in wildtype (WT) littermates (Fig. 1.11) (Rieger, Sims et al. 2010).

In addition to being a downstream target of Wnt signaling during development, \textit{Lbh} was found to be a target of oncogenic Wnt signaling in murine
breast cancer models and in primary human breast tumors (Rieger, Sims et al. 2010) (see section 1.3.7).

1.3.6 LBH in Breast Cancer

Given that Lbh was found to be a direct target gene downstream of normal Wnt signaling and that LBH can act to maintain the undifferentiated progenitor state of numerous tissue types (Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009, Rieger, Sims et al. 2010), our laboratory investigated whether Lbh was expressed in mammary tumors of a Wnt driven tumor model: MMTV-Wnt1 (Tsukamoto, Grosschedl et al. 1988). MMTV-Wnt1 mice develop highly aggressive adenocarcinomas, which have been shown to be enriched in basal K5+/ER- cancer stem cells (Li, Welm et al. 2003, Liu, McDermott et al. 2004, Herschkowitz, Simin et al. 2007, Watanabe, Fallahi et al. 2013). Using in situ RNA hybridization and Western Blot (WB) analysis (Rieger, Sims et al. 2010) together with immunohistochemistry (IHC) (Lindley unpublished), our data collectively demonstrated that Lbh is overexpressed in mammary tumors from MMTV-Wnt1 mice (Fig. 1.12A,B). Furthermore, Lbh overexpression correlated with K5 positivity and K8 negativity (Fig. 1.12C). These data indicate that Lbh is indeed expressed downstream of oncogenic Wnt signaling and suggest an association of LBH with a basal cell phenotype during carcinogenesis.
Analysis of 51 established breast cancer cell lines and 1,107 primary human breast tumors using meta-analysis of published micro-array datasets confirmed that high levels of \textit{LBH} expression are associated with basal subtype tumors (Rieger, Sims et al. 2010). Additionally, \textit{LBH} expression was found to positively correlate with basal \textit{K5} expression and canonical Wnt pathway gene hyper-activation (\textit{SFRP1}, \textit{TCF4}, \textit{TCF7} and \textit{DKK3}) in primary tumors (Rieger, Sims et al. 2010). \textit{LBH} also displayed a strong inverse correlation with expression of prognostic marker \textit{ER\alpha}, thus indicating that \textit{LBH} may serve as a potential biomarker for poor prognosis, difficult to treat, ER-, basal subtype tumors (Rieger, Sims et al. 2010).

\textit{In vitro} analysis of basal subtype, TNBC cell lines revealed that \textit{LBH} overexpression in these tumor cells can be attenuated through inhibition of canonical Wnt signaling by treatment with non-canonical Wnt7a ligand or
recombinant DKK1 protein a Wnt inhibitor (Rieger, Sims et al. 2010). Knockdown (KD) of LBH in TNBC cell lines using RNAi reduced the CD44\textsuperscript{high}CD24\textsuperscript{low/-} BCSC population and subsequent formation of colonies in soft agar (Rieger 2010). Conversely, overexpression of LBH in TNBC cell lines with low levels of endogenous LBH resulted in increased colony formation in soft agar (Rieger 2010).

1.4 Thesis Objective and Hypothesis

Based on both published and preliminary functional data primarily from our laboratory, the main hypothesis this thesis sought to address has been that LBH critically contributes to the maintenance and function of both normal MaSCs as well as BCSCs during oncogenesis. To test this hypothesis, I have: a) determined whether LBH expression in human mammary epithelial cells (HMEC) is regulated by stem cell pathways other than WNT with focus on the TGF\textbeta{} pathway; b) addressed the mechanistic function of LBH in normal MG development \textit{in vivo} using a novel genetic conditional \textit{Lbh} knockout mouse model, and c) assessed the potential role for LBH in generating and maintaining BCSCs by experimentally modulating \textit{LBH} expression levels in established human breast cancer cell lines. Overall, these studies have provided the first mechanistic insight into the hitherto unknown physiological function of LBH suggesting a critical requirement for LBH transcription co-factor function in adult stem cell regulation and malignant cancer stem cell development. These findings
are highly significant, as LBH is potential novel biomarker and therapeutic target for basal subtype TNBC and other WNT-dependent human diseases.
Chapter 2: Material and Methods
# 2.1 Materials

## 2.1.1 Commonly Used Lab Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligonucleotides</td>
<td>PCR Primers</td>
<td>Sigma Genosys</td>
</tr>
<tr>
<td>Cell Culture/Media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mammalian cell lines</td>
<td>Dulbecco’s Modified Eagle’s Medium (DMEM), Roswell Park Memorial Institute -1640 (RPMI 1640), Improved MEM (IMEM)</td>
<td>Mediatech</td>
</tr>
<tr>
<td>Primary cells</td>
<td>DMEM, EpicultB, Hanks balanced salt solution (HBSS)</td>
<td>Mediatech, Stemcell Technologies</td>
</tr>
<tr>
<td></td>
<td>FBS, Heat-Inactivated FBS (HI-FBS)</td>
<td>Gibco, Invitrogen</td>
</tr>
<tr>
<td></td>
<td>Non-essential amino acids (NEAA), Penicillin-streptomycin (Pen/strep), Sodium Pyruvate, Glutamine, G418</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Stem Cell Reagents</td>
<td>poly(2-hydroxyethyl methacrylate)</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td>B27</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td>DNase1, collagenase/hyaluronidase, dispase, NH$_4$Cl,</td>
<td>Stemcell Technologies</td>
</tr>
<tr>
<td>Protein Work</td>
<td>Nitrocellulose membrane</td>
<td>Whatttman</td>
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<tr>
<td></td>
<td>West Fempto Super Signal Western Blot Detection Kit</td>
<td>Pierce</td>
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<tr>
<td></td>
<td>8” x 5” film</td>
<td>Denville, Midwest Scientific</td>
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<tr>
<td></td>
<td>Hoechst Dye</td>
<td>Sigma</td>
</tr>
<tr>
<td></td>
<td>SlowFade mounting media</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Plastics</td>
<td>6-well plates, dishes, chamber slides</td>
<td>VWR, BD</td>
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<tr>
<td>Transfection</td>
<td>Dharmafect 1-4</td>
<td>Dharmacon</td>
</tr>
<tr>
<td></td>
<td>ON-Target smart pool siRNA</td>
<td>Dharmacon</td>
</tr>
</tbody>
</table>

**Table 2.1:** general lab reagents used in this thesis.
2.1.2 Mammalian cell lines

All cell lines were obtained from ATCC with the exception of HMEC, which were obtained from Cambrex. Primary cells were isolated from fresh dissected mammary glands of 8 week old virgin mice. Cells were cultured in an atmosphere of 5% CO2 at 37°C. Cell lines with appropriate culture conditions are listed in Table 2.2.

<table>
<thead>
<tr>
<th>Cell Line Name</th>
<th>Culture Conditions</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC11</td>
<td>RPM1 1640, 10% FBS, 10 ng/ml EGF, 5 μg/ml insulin, 1% pen/strep</td>
<td>Mouse non-transformed mammary epithelial cells</td>
</tr>
<tr>
<td>HMEC</td>
<td>HuMEC basal serum free media and supplement kit</td>
<td>Human primary mammary epithelial cells</td>
</tr>
<tr>
<td>MCF10A</td>
<td>HuMEC basal serum free media and supplement kit</td>
<td>Human non-transformed mammary epithelial cells</td>
</tr>
<tr>
<td>MCF7</td>
<td>IMEM, 10% FBS, 10μg insulin, 1% pen/strep</td>
<td>Human breast adenocarcinoma, pleural effusion derived cell line</td>
</tr>
<tr>
<td>BT-549</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast adenocarcinoma, pleural effusion derived cell line</td>
</tr>
<tr>
<td>HCC1187</td>
<td>RPM1 1640, 10% FBS, 1% pen/strep</td>
<td>Human breast ductal carcinoma cell line</td>
</tr>
<tr>
<td>MDA-MB-435S</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast ductal carcinoma, pleural effusion derived cell line</td>
</tr>
<tr>
<td>MDA-MB-157</td>
<td>DMEM, 10% FBS, 1% NEAA, 1% pen/strep</td>
<td>Human breast medullary carcinoma</td>
</tr>
<tr>
<td>Breast Cancer Cell Lines*</td>
<td>DMEM no phenol red, EGF 20ng/ml, pen/strep 1mg/ml, B27 supplement</td>
<td>See specific details for each cell line</td>
</tr>
<tr>
<td>Primary Mouse Mammary Epithelial Cells*</td>
<td>HuMEC, supplements, 5% FBS</td>
<td>Mouse mammary epithelial cells, isolated from freshly dissected mammary glands</td>
</tr>
<tr>
<td>Primary Mouse Mammary Epithelial Cells**</td>
<td>DMEM, DMEM/F12 with phenol red, EGF 20ng/ml, FGF 20ng/ml, Heparin 4μg/ml, pen/strep 1mg/ml, and B27 supplement</td>
<td>Mouse mammary epithelial cells, isolated from freshly dissected mammary glands</td>
</tr>
</tbody>
</table>

Table 2.2: Mammalian cell lines and primary cell culture conditions. *Indicates culture media for growth in differentiation assays. **Indicates media for mammosphere culture.
## 2.1.3 Antibodies and Recombinant Protein

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Source</th>
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</tr>
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<tbody>
<tr>
<td>Rabbit polyclonal LBH 6th bleed affinity purified</td>
<td>In house</td>
<td>WB, IF, IHC, IP</td>
</tr>
<tr>
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<td>Rabbit polyclonal Ki67</td>
<td>Novus</td>
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<tr>
<td>Rabbit polyclonal PR</td>
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Table 2.3: Antibodies and recombinants proteins.

2.1.4 Oligonucleotide Sequences

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**Genotyping primers**

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<td>Lbh loxP allele (NeoΔ2)</td>
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**Table 2.4:** qPCR and genotyping oligonucleotide sequences.
2.2 Methods

2.2.1 Cell Culture and Tissue Dissociation

*TGFβ Induction Studies*

For EMT induction, cells were pre-seeded at a density of $1.5 \times 10^4$ cells/cm² for 24 h and recombinant human TGFβ1 (R&D Systems) was added to media at a concentration of 5 ng/ml. Partial media changes supplementing fresh TGFβ1 were performed every other day. TGFβ1-treated HMEC were split multiple times in parallel with untreated control cells once the latter reached confluency.

*Knockdown (KD) by RNAi*

For RNAi studies, cells were transfected with 100 nM of synthetic siRNA specific for *TPp63*, *LBH*, or a scrambled control sequence using Dharmafect reagent (Dharmacon). Cells were incubated with siRNA containing media for 72 hours prior to collection for downstream applications (mammospheres, transwell migration, transwell invasion) and analysis (RNA and protein). KD efficiency was determined by collecting total RNA, which was isolated using TRIzol® Reagent (Invitrogen).

*Isolation of Primary MECs and Dissociation into Single Cell Suspension*

MGs were dissected from female mice at 8 weeks of age. Tissue was minced with a scalpel and placed in dissociation medium (EpicultB + supplements, EGF 20ng/ml, FGF 20ng/ml, Heparin 4μg/ml, 5% heat-inactivated FBS [HI-FBS], 10x Collagenase/Hyaluronidase) ~3ml per mouse in 15ml conical vial. Minced glands were allowed to dissociate for 4-5 hrs at 37° C with agitation.
The organoid suspension was then treated sequentially with 1) 5ml 0.8% NH₄Cl + 0.1mM EDTA in Hank’s Balanced Salt Solution (HBSS) and gentle pipetting to lyse red blood cells - 1 minute 2) 8ml of 0.25% Trypsin-EDTA with vigorous pipetting - 3 minutes, followed by 3) neutralization with 8ml HBSS + 2% HI-FBS and 4) 2ml dispase 5mg/ml + DNase 1mg/ml with gentle pipetting for 1-2 minutes (cells are spun down for 5 minutes at 450g between each of steps 1-4; though additional time is sometimes needed after step 3). The suspension from step 4 was diluted in 8ml's of HBSS + 2% HI-FBS, filtered through a 40µm filter incrementally in 1ml volumes and then spun down (450g) and further mechanically dissociated using a 25 gauge needle and syringe (cells were resuspended in 1ml of PBS+2% HI-FBS prior to being pulled through the syringe 5 times). Cells were visualized using microscopy to ensure a single cell suspension and counted using a hemocytometer for downstream applications.

**Preparation of Single Cell Suspension - Cell Lines**

Cells were trypsinized and neutralized with appropriate FBS containing culture media followed by spinning down of the cell pellet (450g) and resuspension in 1ml ice-cold PBS. Cells were then filtered through a 70µm filter to remove large aggregates and debris. Mechanical dissociation using a 25 gauge (gauge size adjusted based on cell type) needle and syringe was performed (5+ times) and visualized using microscopy to ensure complete dissociation to single cell suspension.
**Mammosphere Culture**

Single cell suspensions of breast cancer cell lines, normal MEC lines or primary MECs were prepared and counted. Cells were then plated in triplicate wells of a 6 well plate at a density of: 2-5 x 10³, 5 x 10³, or 1-2 x 10⁴ cells per well respectively (2ml/well). 6-well plates were pre-coated with 12mg/ml poly(2-hydroxyethyl methacrylate) (Sigma) in 95% ethanol, and baked at 48° C for 48hrs. Cells were grown in mammosphere media (as indicated in Table 2.2): 7-10 days (cell lines); 10-14+ days (primary cells). Primary spheres larger than 50µm in size were counted and quantified. Spheres could then be enzymatically and mechanically dissociated to a single cell suspension and re-plated to assess secondary sphere formation if desired.

**Dissociation of Primary Spheres for Secondary Culture**

Culture media including spheres was collected and wells washed with 1ml of PBS. Media and spheres were then spun down/pelleted (450g) and resuspended with 300-400µl of 0.25% Trypsin EDTA and incubated at 37° Celsius for 3 minutes. Spheres were then mechanically dissociated using a 25 gauge syringe; taking caution with the degree of dissociation and the cell type as primary cells are inherently more fragile. Trypsin was then neutralized with 2% FBS containing media and cells spun down (450g) and resuspended in PBS for counting and replating in mammosphere culture.

**Mammosphere Differentiation Assay**

After growth in mammosphere culture for 10-14 days, spheres generated from primary MECs are picked with a 20µl pipette tip and transferred to collagen
coated 8-well chamber slides (~5 spheres per well; BD). A mixture of 0.1% collagen (w/v) dissolved in 0.1M acetic acid is diluted 1:10 with water and used to pre-coat chambers slides for 1 hour at room temperature. Excess collagen mixture is removed and chamber slides used immediately or stored for up to one month at 4º C. Spheres are grown in 2D culture in differentiation medium: HuMEC, +supplements, 5%FBS (Invitrogen) for 5 days before assessment with downstream application i.e. immunofluorescence see section 2.2.4.

**In Vitro Migration Assay**

Cells were typsinized and plated on the 6.5mm Transwell® Inserts with 8.0µm Pore Polycarbonate Membrane (Corning) on a 24 well plate in 150 µl serum free medium. Approximately 750µl of appropriate medium (see table 2.2) with 10% FBS (Invitrogen) was added in the same well as a chemo-attractant. 12-14 hours later, media was removed and inserts were washed 3 times with 500µl PBS. Before fixation, the cells that didn’t migrate through the membrane were wiped off with PBS moistened cotton tips and the inserts were fixed with 100% methanol for 5 minutes followed by staining with crystal violet (Invitrogen) for 5 minutes. The inserts were rinsed with tap water briefly and membranes of the inserts were taken off by a fine tip blade. Membranes were then mounted on glass slides and images from 3 randomly chosen fields were taken followed by quantification of those cells that had migrated.

**In Vitro Matrigel Invasion Assay**

Cells (1 - 2 x 10^4) were resuspended in 100 µl of serum-free growth medium and plated onto Matrigel-coated 8.0-µm transwell filter inserts (Corning)
of 24-well plates in triplicates. Filters were pre-coated with 10 µl of matrigel (BD) diluted 1:4 in ice-cold serum-free medium (table 2.2) and allowed to solidify for 1 h at 37°C before use. The bottom wells contained 500 µl of growth medium with 10% FBS as a chemo-attractant. After incubation for 48 hours, cells on the upper surface of the filter were removed with a cotton swab and cells on the bottom side were fixed in 100% methanol and stained with 1% Toluidine Blue in 1% Borax (Invitrogen). Membranes were then mounted on glass slides and images from 3 randomly chosen fields were taken followed by quantification of those cells that had migrated.

2.2.2 Fluorescence Activated Cell Sorting (FACS)

**FACS Analysis of Human Normal MEC and Breast Cancer Cell Lines**

Between 2 × 10⁵ and 1 × 10⁶ cells were resuspended in 100 µl FACS buffer (PBS, 2% FBS, 0.1% Sodium Azide), and incubated for 20 min in the dark with 20 µl CD44-APC and CD24-PE antibodies (BD Pharmingen; table 2.3) on ice. Cells were washed in 3 ml FACS buffer and resuspended in a final volume of 500 µl FACS buffer for analysis. Unstained, and single antibody samples were used to calibrate the analyzer for each experiment. FACS analysis was performed on a FACS Calibur II (BD) or LSR-I (BD) flow cytometric analyzer and analyzed with FlowJo software.

**FACS Analysis of Primary Mouse MECs**

Approximately 2x10⁶ MECs per sample were blocked 10 minutes with anti-CD16/CD32 and Rat-γ- globulin (1µl/10⁶ cells; BD Biosciences; Jackson Immuno
Research). Cells were split into tubes for unstained, single color, and live/dead cell controls and then stained individually or in combination with anti-CD24-PE (1µl/10^6 cells), anti-CD29-FITC (1µl/10^6 cells) (BD Biosciences; Serotec), and APC-conjugated CD45, CD31 and TER119 antibodies (1µl/10^6 cells each; BD Biosciences) in 50µl of PBS+2% HI-FBS for 30 minutes. Cells were then washed and incubated 30 minutes with Streptavidin-APC (1µl/10^6 cells; Invitrogen) and violet dead cell marker (0.5µl/10^6 cells; Invitrogen) in 50µl of PBS+2% HI-FBS. Cells were filtered through a 40µm filter before FACS sorting on a FACSARia-IIu (BD) sorter followed by analysis with FlowJo software. Cells were collected for gene expression analysis using qPCR or for downstream functional assays (mammosphere).

2.2.3 Quantitative Real-Time PCR (qPCR)

Purification of total RNA was performed using TRIzol® Reagent (Invitrogen) followed by Turbo-DNase treatment (Ambion). cDNA was synthesized from DNase-treated total RNA using Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Promega). qPCR reactions were carried out with 50–100 ng of DNase-treated cDNA, and 2 µM of gene-specific primers (Table 2.4) in a 20 µl reaction containing SsoFast EvaGreen Supermix (NEB). PCR amplification was performed with a 30 second hot start at 95 °C, and 40 cycles of: 5 seconds at 95 °C followed by 10 seconds at 60 °C. Reactions were performed in triplicates on a CFX96 thermocycler (Biorad). Melting curves were included for every run to ensure that only one correct product was
amplified. The Ct values were normalized to the corresponding values of GAPDH. Normalized data points were plotted in standard or log-scale as noted. Error bars on the graphs represent the standard error of the mean of each sample in triplicates.

2.2.4 Immunofluorescence (IF)

Cells were grown overnight on BD Bioscience Culture Slides seeded at an appropriate density such that ~70% confluency was reached the day of the experiment. Cells were fixed with 2% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature, followed by cell permeabilization in 0.1-0.3% Triton X-100 in PBS depending on cellular localization of the protein that was to be visualized (higher concentrations of TritonX-100 for nuclear proteins etc). Cells were blocked for 1 hour at room temperature in PBS plus 10% Normal Goat Serum (NGS) in a humidified chamber. Antibodies were incubated for 1 hour at room temperature (Lbh 6th bleed affinity purified -1:200, Keratin 5 (Covance) - 1:5,000, Keratin 8/18 – 1:100, phalloidin-FITC (Sigma)- 1:300, E-cadherin (BD) - 1:1000, ZO-1 (Zymed)- 1:500, Vimentin (Sigma)- 1:500 , Fibronectin (Hybridoma Bank)- 1:2500, in 10% NGS. This incubation was followed by washing and a subsequent one hour incubation in the dark with the appropriate antibodies in 10% NGS (anti-mouse Cy3 - 1:400, anti-rabbit FITC – 1:200, anti-mouse or rabbit Alexafluor 488 or 594 - 1:500, anti-guinea pig Alexafluor 568 – 1:500). After sufficient washing, coverslips were mounted with Slowfade + DAPI (Molecular Probes) according to the manufacturer's protocol. If no DAPI was
included, Hoechst dye (Sigma) was included at a dilution of 1:10,000 in the first wash after the secondary antibody incubation to stain the nuclei.

2.2.5 Histology and Immunohistochemistry

*Immunohistochemistry (IHC) Analysis*

Tissues were fixed in 4% (wt/vol) PFA overnight, dehydrated in ethanol, embedded in paraffin, and sections of 5 µm were prepared. Tissue sections, after de-paraffinization and rehydration, were subjected to antigen retrieval by boiling in 0.1 M Na citrate pH 6.2 or 10mM Tris/ 1mM EDTA pH 9.0 for 10 min in a decloaking chamber (BioCare Medical). Endogenous peroxidase activity was quenched for 30 min at RT with 0.3% Hydrogen Peroxide in PBST (PBS plus 0.1% Triton X100), followed by blocking for 1h in PBST containing 10% normal goat serum/NGS (Sigma). Sections were incubated overnight at 4°C with antibodies: affinity-purified LBH antibody (1:75-100), Keratin 5/6 (Covance)-1:5000, Keratin 8 (Hybridoma Bank)- 1:500, Estrogen Receptor (SantaCruz)-1:300, Progesterone Receptor (Santa Cruz)- 1:300, Ki67 (Novus)- 1:300, ΔNp63 (Biolegend)- 1:500, TAp63 (Biologend)- 1:500, in PBST-1% NGS. Secondary HRP-coupled antibodies (1:500; Invitrogen) were added for 1h at RT followed by detection of immunostaining with Vector NovaRED substrate (Vector Labs) and counterstaining of sections with hematoxilin.

*Hematoxilin and Eosin (H&E) Histological Staining*

Tissues were fixed in 4% (wt/vol) paraformaldehyde (PFA) overnight, dehydrated in ethanol, embedded in paraffin, and sections of 5 µm were
prepared. Tissue sections, after de-paraffinization and rehydration were stained with eosin; followed by hematoxylin. Slides were then dehydrated and mounted using Permount (Fischer Scientific).

2.2.6 Mammary Gland Whole Mount Analysis

Whole inguinal mammary glands (#4) were dissected and spread onto a glass slide, allowed to dry for 1 min, and fixed 2-4hrs in Carnoys fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid). The glands were then stained in carmine alum solution (1 g of carmine red [Sigma], 2.5 g of aluminum potassium sulfate [Sigma], 500 ml of dH2O) overnight. The tissues were destained in 70% ethanol and cleared in Xylene (VWR). Images of stained glands were taken on a Leica MZ16 stereoscope equipped with a Q-capture imaging camera.

2.2.7 Western Blot

Cells extracts were either collected or lysed directly on the plate in SDS sample buffer (125 mM Tris-HCl pH6.8, 10% glycerol, 2% SDS, 0.0005% Bromophenol blue). Protein concentration was measured by BCA assay (Pierce). A total of 15-25 µg protein extract per sample was separated by SDS-PAGE, blotted on nitrocellulose membrane and incubated with the following antibodies in TBST (20 mM Tris HCl pH 7.5; 140 mM NaCl, 0.1% Tween 20) plus 5% non-fat dry milk: affinity purified Lbh 6th bleed (1:1000), Keratin 5/6 (Covance; 1:10,000), Keratin 8/18 (Progen, 1:2,000), ERα (Santa Cruz, 1:1,000), E-cadherin (BD;
1:2500), ZO-1 (Zymed; 1:1000), N-Cadherin (BD; 1:200), Vimentin (Sigma; 1:50,000), Fibronectin (Hybridoma Bank; 1:5000), ΔNp63 (Biolegend; 1:1000), β-actin (Sigma, 1: 10,000), and anti-rabbit, anti-mouse, or anti-guinea pig HRP coupled secondary antibodies (1:25,000). Signal was detected using the West Fempto Super Signal kit (Pierce).

2.2.8 Affinity Purification of LBH Antibody

Recombinant HPLC-purified LBH protein (10µg) (Al-Ali, Rieger et al. 2010) was separated on a 15% SDS-PAGE gel and transferred to BA85-nitrocellulose membrane (Schleicher-Schuell). The nitrocellulose membrane was stained with Ponceau Red (Sigma) to facilitate visualization and excision of the LBH-specific protein band. The LBH protein-containing nitrocellulose strip was treated with 100 mM glycine/HCl pH 2.5 for 5 min and washed 2 x 2 min in TBS (20 mM Tris-HCl pH7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM CaCl2, 0.5 mM MgCl2, 0.6 mM Na2HPO4) to remove excess unbound protein. After blocking in TBS plus 3% BSA, the membrane was incubated overnight with 50 µl of rabbit polyclonal LBH anti-serum diluted in 350 µl TBS. Unspecific bound IgG’s were removed by washing with TBS 3 x 5 minutes. LBH-specific IgG’s were then eluted by applying 30µl of 100 mM glycine/HCl pH2.5 to the nitrocellulose strip at RT for 10 minutes. A small whole was punched in the membrane containing tube so that eluate could be spun into a collection tube with a volume of 10 µl of 1M Tris pH 8.0. This elution step was again repeated with 10µl of 100 mM glycine/HCl pH2.5 for 10 minutes at RT and spun into the collection tube containing the previous eluate.
and Tris pH 8.0. Sodium azide and BSA were added at a final concentration of 5 mM and 1mg/ml respectively to maintain antibody stability.

### 2.2.9 Mouse Techniques

**Gene targeting and generation of conditional Lbh mutant mice**

A 129/SvEv Bacterial Artificial Chromosome (BAC) library was screened for *Lbh*-specific genomic BAC clones. The accuracy of restriction enzyme sites and exon/intron boundaries listed on the Ensemble Mouse Genome Server (http://www.ensembl.org/Mus_musculus/) for a C57BL/6 genetic background were confirmed by DNA sequencing of genomic clones and mapping of the endogenous *Lbh* genomic locus by restriction enzyme and Southern blot analysis using genomic DNA from W4 129/SvEv embryonic stem (ES) cells and *Lbh*-specific cDNA probes (Briegel and Joyner 2001). A targeting vector in a pSP72 (Promega) vector backbone was constructed by inGenius Targeting Laboratories by first subcloning a 11.0 kb *Lbh*-specific genomic region from 129/SvEv BAC clone RP22: 54C4 that contained sequences 5,176 bp upstream of the transcriptional start site, exon 1, intron 1, targeted exon 2, and 3,206 bp downstream of exon 2 using BAC recombineering in *E. coli* (Yang, Musci et al. 1997, Misulovin 2001). A PGK-Ptr/loxp-P-Neo cassette was inserted 220 bp downstream of exon 2, which created a 5’ homologous arm of ~8.0 kb (8,030 bp) and a 3’ homologous arm of ~3.0 kb (2,986 bp) flanking Neo. A single loxP site was inserted into the 5’ homologous arm in intron 1 ~1.7 kb upstream of exon 2. After linearizing with *Ascl*, the targeting construct was electroporated into
129/SvEv ES cells (inGenious), and clones with a homologous recombination event were selected in media containing G418 (Matise 2001). Three correctly targeted ES cell clones were identified among 288 neomycin resistant colonies screened (~1% targeting frequency) using PCR and Southern Blot analysis. Two clones (#233 and #644) were injected into blastocysts from C57BL/6 females to produce chimera (Hogan et al., 1994), and a colony of $Lbh^{+/flox}$ mice was successfully established from clone #233.

To remove the Neo cassette, $Lbh^{+/flox}$ mice were crossed with ROSA26-$FLPe$ deleter mice (129S4/SvJaeSor-Gt(ROSA)26Sor$^{tm1(FLP1)Dym}$/J; strain; Jackson Laboratories; stock #003946) (Farley, Soriano et al. 2000), generating offspring with a conditional $Lbh^{loxP}$ allele. To produce mice with a germ line deletion of exon 2, $Lbh^{+/loxP}$ mice were mated with ROSA26-Cre deleter mice (C57BL/6-Tac-Gt(ROSA)26Sor$^{tm16(Cre)Arte}$; Taconic-Artemis, stock #6467) (Otto, Fuchs et al. 2009). Mice have been maintained on a hybrid 129/Sv x C57BL/6 background and are available upon request from KJB. These studies followed the recommendations of The Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and were approved by the University of Miami Institutional Animal Care and Use Committee (Protocol #11-060).

Genotyping

Genomic DNA was isolated from ES cell clones and mouse tails, and analyzed by Southern blot analysis (Matise et al., 2001). Probes were generated by genomic PCR using the following primer pairs: PB1, 5’-ATCTGATTCCAAGCGTGCTTGTGCTT-3’ and PB2, 5’-
ACAGGAATGCACTTCTGCAGCC–3’ to obtain a 513 bp 5’ external probe (P1); PB3, 5’-TCGCCAAATGGTATTTACTAGGC-3’ and PB4, 5’-AGTAAAAGGTCCACCAACATGG-3’ to obtain a 790 bp 3’ internal probe (P2).

Mice carrying the wild-type Lbh+ mice allele (394 bp) and the targeted Lbhfloxd and LbhloxP alleles (456 bp) were genotyped by PCR using tail genomic DNA and the primer pair f1 (LOX1), 5’-TCTGCTTAGCCAGAGACTG-3’ and r1 (SDL2) 5’-CAAGGCCTCCACCACCTTCAAAG-3’ that flank the 5’loxP site upstream of exon 2. Deletion of Neo was genotyped by PCR using the primer pair f2 (Neo Del3), 5’-TCAGCCCCCGCAAGGATGCC-3’ and r2 (Neo Del2), 5’-TCACCCTGAGGACAGGTCCT-3’ resulting in a 420 bp amplicon from the wild-type Lbh+ allele and a 595 bp amplicon from the Neo-deleted LbhloxP allele, which contained additional single Flt-loxP sites. Mice carrying the Lbh∆2 null allele (358 bp) were genotyped with a triple PCR primer set consisting of f1 and f2 as forward primers and r2 as reverse primer. The presence of FLPe was verified by PCR with the primer pair FLP1, 5’-CAGGGTTAGTCAGATGTTG-3’ and FLP2, 5’-CTAGTGCAAGTATGAGTCCAGG-3’ resulting in a ~800 bp amplicon; and of Cre with the primer pair oIMR1084, 5’-CAGGGTTAGTCAGATGTTG-3’ and oIMR1085, 5’-GTGAAACAGCATTGTCATTTG-3’ resulting in a ~100 bp amplicon. PCR reactions were carried out on a BioRad thermocycler using Novagen Taq polymerase; 95°C for 5 minutes; 40 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and one additional cycle at 72°C for 7 min.
Quantitative Real-Time PCR (qPCR) of Lbh Transcript in Lbh$^{\Delta 2/\Delta 2}$ Mice

qPCR primer sets were: f3 (forward-exon 1), 5'-CCCTGCCAATCTCATTCAAGGAC-3' and r3 (reverse-exon 3), 5'-CTTCAGTGGGTCCACCAC-3' to amplify sequences spanning exons 1 to 3 in the wild-type Lbh$^+$ allele (326 bp) and the Lbh$^{loxP}$ null allele (223 bp); and f4 (forward-exon2): 5'-GAGATCGGCTGAGATGACC-3' in combination with r3 to amplify sequences spanning exons 2 to 3 in the wild-type Lbh$^+$ allele (173 bp) and resulting in no product in the Lbh$^{\Delta 2}$ allele.

Statistical Analyses

The statistical significance of deviations from expected Mendelian ratios (1:2:1) of offspring from R26-Cre:Lbh$^{+/loxP}$ intercrosses was determined by Chi-square tests using GraphPrism software. All other statistical analysis was performed using unpaired two-tailed Student’s t-test. A P-value of <0.05 was considered to be significant.
In Vivo Role of LBH in Mammopoiesis

Chapter 3: Generation of Mice with a Conditional Lbh Null Allele*

*A version of this chapter is in press at Genesis (Lindley and Briegel 2013)
3.1 Background

Limb-Bud and Heart (LBH) is a highly conserved, tissue-specific transcription cofactor in vertebrates with no homology to known protein families (Ai, Wang et al. 2008, Al-Ali, Rieger et al. 2010) (Briegel and Joyner 2001, Briegel, Baldwin et al. 2005). Recent work has shown that Lbh is a direct target gene of the WNT signaling pathway (Rieger, Sims et al. 2010), a genetic network fundamental to embryonic development, adult tissue homeostasis, and oncogenesis (Logan and Nusse 2004). During mouse embryonic development, LBH is expressed after gastrulation with a unique spatio-temporal gene expression pattern in the embryonic limb buds, heart, gut, kidney, gonads, peripheral nervous system, brain, and bones (Briegel and Joyner 2001, Briegel, Baldwin et al. 2005, Hoogaars, Engel et al. 2007, Conen, Nishimori et al. 2009). Aberrant gain-of-function of LBH, which maps to chromosome 2p23, is associated with partial trisomy 2p syndrome (Briegel, Baldwin et al. 2005), a rare human autosomal disorder characterized by multiple congenital anomalies including cardiovascular, skeletal and postaxial limb malformations (Lurie, Ilyina et al. 1995). Modeling of pathologic LBH gain-of-function during embryonic heart development through overexpression of a cardiac-specific CARP-Lbh transgene in mice phenocopied congenital heart disease observed in partial trisomy 2p syndrome patients (Briegel, Baldwin et al. 2005). Moreover, retroviral overexpression of LBH in chick embryonic limbs resulted in abnormal skeletal growth and delayed bone differentiation (Conen, Nishimori et al. 2009), suggesting that aberrant LBH gain may also contribute to skeletal defects in
these patients. Our previous work demonstrated that Lbh is expressed during specific stages of postnatal mammary gland development, whereas it is abnormally overexpressed in aggressive, treatment-resistant basal-subtype breast cancers correlating with pathologic WNT pathway hyperactivation (Rieger, Sims et al. 2010). However, the normal functioning of Lbh in organogenesis is presently unknown due to the lack of an Lbh knockout mouse or other genetic loss-of-function models.

To address this we have generated mice with a conditional null allele of Lbh by flanking exon 2 with loxP sites (Lbh<sup>flox</sup>). Homozygous Lbh<sup>flox</sup>, and Lbh<sup>loxP</sup> mice, in which the Neo cassette was removed through FLPe-mediated recombination, were viable and fertile, indicating that these conditional Lbh alleles are fully functional. Lbh<sup>loxP</sup> mice were then crossed with a Rosa26-Cre line resulting in ubiquitous deletion of exon 2 and abolishment of LBH protein expression. Mice homozygous for the Lbh null allele (Lbh<sup>Δ2</sup>) displayed normal embryonic development and postnatal growth with morphologies indistinguishable from wild-type littermates. However, mammary gland development, which occurs primarily after birth, was perturbed. Thus, the conditional Lbh allele will be a valuable tool to uncover the currently unknown tissue-specific roles of LBH in postnatal development and disease.

### 3.2 Targeting Strategy

To elucidate the in vivo roles of LBH in development and disease, we have generated mice harboring a Lbh conditional null allele based on the Cre-
$\textit{loxP}$ and $\textit{Flp-Frt}$ systems (Nagy 2000, Rodriguez, Buchholz et al. 2000) and using homologous DNA recombination in murine embryonic stem (ES) cells (Matise 2001). The murine $\textit{Lbh}$ locus encompasses a region of approximately 30 kb, and contains 3 exons encoding a mRNA of 3,086 nucleotides and a protein of 105 amino acids (Briegel and Joyner 2001) (Fig. 3.1a, 3.2a). A conditional allele ($\textit{Lbhflox}$) was generated by inserting unidirectional $\textit{loxP}$ sites 5’ and 3’ of exon 2 (Fig. 3.1a), such that Cre-mediated recombination would result in the deletion of exon 2 (amino acids 9 – 43), producing an $\textit{Lbh}$ null allele ($\textit{Lbh}^\Delta_2$) (Fig. 3.1a). Splicing of exon 1 to 3 produces a conceptual frame shift, introducing an early stop codon that removes most of the LBH coding sequence including the nuclear localization signal and a C-terminal glutamate-rich trans-activation domain (Briegel and Joyner 2001) (Fig. 3.2a). (For a more detailed description of the targeting strategy please see Methods section 2.2.8).

### 3.3 Germline Transmission of $\textit{Lbhflox}$ Allele

After gene targeting in 129/SvEv ES cells, neomycin-resistant clones were screened by Polymerase Chain Reaction (PCR) and Southern blot analyses (Fig. 3.1b; data not shown). Insertion of the 5’$\textit{loxP}$ site introduced an additional EcoRI site into the targeted $\textit{Lbh}$ locus, and insertion of an $\textit{Frt-loxP}$-flanked Neomycin (Neo) cassette downstream of exon 2, added an additional Ncol site to facilitate identification of the mutant $\textit{Lbhflox}$ by genomic screening (Fig. 3.1a). An external 5’ probe (P1) coupled with an EcoRI digest, and an internal 3’probe (P2) coupled with an Ncol digest detected the wild type (WT) and the $\textit{Lbhflox}$ mutated allele in
correctly targeted events (Fig. 3.1a,b). Male chimeras obtained from correctly targeted ES cell clones were crossed with C57BL/6 females and offspring were screened by PCR analysis of genomic tail DNA to confirm germ line transmission of the \( \text{Lbh}^{\text{flox}} \) allele (Fig. 3.1a, c).

**Figure 3.1:** Generation of the conditional \( \text{Lbh} \) null allele. (a) Schematic representation of the mouse \( \text{Lbh} \) genomic locus and targeting strategy used to produce the \( \text{Lbh}^{\text{flox}} \) conditional allele. The wild type (WT) \( \text{Lbh} \) locus encompasses three coding exons with white boxes indicating the 5’ and 3’-untranslated regions and black boxes the LBH protein coding sequences. The targeting vector contained a 5’ arm with sequences homologous to 8.0 kb of \( \text{Lbh} \) including exons 1 and 2 and a \( \text{loxP} \) site 5’ of the exon 2 to be targeted, a PGK-Neo cassette flanked by tandem \( \text{Frt-loxP} \) sites, and a 3’ arm with sequences homologous to 3 kb of \( \text{Lbh} \) downstream of exon 2. \( \text{LoxP} \) sites are denoted by black triangles, \( \text{Frt} \) sites by white triangles. Additional EcoRI (RI) and Ncol (N) restriction sites in the targeting vector 5’ and 3’ of exon 2 facilitated genomic screening of ES cells. Homologous recombination in 129/SvEv ES cells resulted in the floxed \( \text{Lbh}^{\text{flox}} \) allele. Recombination of the \( \text{Frt} \) sites flanking the Neo selection marker after breeding \( \text{Lbh}^{\text{flox}} \) mice with \( \text{ROSA26-FLP1} \) mice resulted in the \( \text{Lbh}^{\text{loxP}} \) allele, and recombination of the \( \text{loxP} \) sites flanking exon 2 after breeding with \( \text{ROSA26-Cre} \) mice in the \( \text{Lbh}^{\Delta 2/\Delta 2} \) null allele. The location of genomic probes (black rectangles) and primers for PCR genotyping (black arrows) is indicated. (b) Southern blot analysis was performed on genomic DNA from neomycin-resistant 129/SvEv ES cell clones to screen for homologous recombinants. A 5’ external probe (P1) was used after EcoRI digest to distinguish a 28.3 kb band of the wild type (WT) allele from a 8.5 kb band of the mutant \( \text{Lbh}^{\text{flox}} \) allele. Additionally, a 3’ internal probe (P2) was used after Ncol digest to distinguish a 4.7 kb WT band from a 5.2 kb band of the mutant \( \text{Lbh}^{\text{flox}} \) allele. (c-e) PCR analysis using mouse tail genomic DNA was performed to identify (c) germ line transmission of the \( \text{Lbh}^{\text{flox}} \) allele, (d) \( \text{Frt} \)-mediated excision of Neo (\( \Delta \text{neo} \)), and (e) \( \text{Cre} \)-mediated excision of exon 2 (\( \Delta \text{E2} \)) in mice. The PCR primers used and the corresponding sizes of the amplification products are indicated.
3.4 Generation of Mice Harboring an \textit{Lbh}^{Δ2} Null Allele

To remove the Neo cassette, heterozygous offspring (\textit{Lbh}^{+/\textit{flox}}) were crossed with \textit{ROSA-FLPe}-deleter mice (Farley, Soriano et al. 2000). Successful deletion of Neo, generating the \textit{Lbh}^{\textit{loxP}} conditional allele, was confirmed by PCR (Fig. 3.1a,d). Homozygous \textit{Lbh}^{\textit{flox/lox}} and \textit{Lbh}^{\textit{loxP/loxP}} mice were viable, fertile, and displayed no anomalous phenotypes relative to their respective heterozygous and WT littermates, suggesting that these conditional \textit{Lbh} alleles are fully...
functional and the insertion of loxP sites and the other foreign genetic elements did not have any position effects.

To assess whether the $Lbh^{loxP}$ allele could be converted to the $Lbh^{Δ2}$ null allele by Cre recombination, we crossed $Lbh^{+/loxP}$ mice with mice expressing Cre ubiquitously from the endogenous ROSA26 promoter (Otto, Fuchs et al. 2009), hereafter referred to as ROSA26-Cre mice. Offspring carrying the correctly recombined $Lbh^{Δ2}$ allele were effectively identified by PCR analysis (Fig. 3.1a,e). Heterozygous $Lbh^{+/Δ2}$ mice were then intercrossed to examine the nature of the conditional $Lbh$ null mutation. We first confirmed that exon 2 was deleted in transcripts produced from the $Lbh^{Δ2}$ allele using quantitative reverse transcriptase-PCR (qPCR) on mRNA from embryos of the different genotypes at embryonic day 10.5 (E10.5). A set of primers complementary to coding

Figure 3.2: Confirmation of conditional $Lbh$ gene inactivation in mice. (a) Schematic representation of mRNA transcripts formed from the wild type (WT) $Lbh$ and $Lbh^{Δ2/Δ2}$ mutant null alleles. The WT transcript encompasses coding exon 1 (amino acids 1-8), exon 2 (amino acids 9-43), and exon 3 (amino acids 44-105), which before splicing are separated by 2.6 kb and 18 kb introns respectively. Cre-mediated excision of exon 2 results in a mutant transcript in which exon 1 is spliced to exon 3, introducing a frame shift and premature stop codon at the exon 1/3 boundary of the $Lbh^{Δ2}$ transcript. This mutation produces a truncated protein of 8 amino acids (AA), which is expected to be non-functional. White boxes = 5' or 3'-untranslated regions; black boxes = LBH protein coding sequences; grey box = coding sequences rendered non-coding by frameshift after Cre-mediated deletion of exon 2. The positions of exon-specific PCR primers are indicated by arrows. (b, c) qPCR analysis of $Lbh$ mRNA expression in WT, heterozygous, and homozygous $Lbh^{Δ2}$ mutant embryos at embryonic day 10.5 (E10.5). (b) Primer sets complementary to sequences in exons 1 and 3 (f3, r3) result in the production of a shorter transcript lacking exon 2 ($ΔE2$) in heterozygous and homozygous mutants. (c) Primer sets complementary to sequences in exons 2 and 3 (f4, r3) fail to amplify a band in homozygous mutants, confirming the deletion of exon 2. (d) Western Blot Analysis using affinity purified LBH-specific antibody shows the absence of LBH protein expression in homozygous $Lbh^{Δ2/Δ2}$ mutant embryos (embryonic day 10-10.5) and reduced LBH protein levels in heterozygous $Lbh^{+/Δ2}$ embryos as compared to WT $Lbh^{+/+}$ littermates. Actin was used as a loading control. The molecular weights (MW) of protein bands are indicated.
sequences in exon 1 and 3 identified a shortened transcript lacking sequences encoded by exon 2 in \( Lbh^{+/\Delta2} \) and \( Lbh^{\Delta2/\Delta2} \) samples, which was absent in WT \( Lbh^{+/+} \) littermates (Fig. 3.2a, b). In addition, no transcripts from exon 2 could be detected by qPCR with exon 2/3-specific primer pairs on cDNA from \( Lbh^{\Delta2/\Delta2} \) embryos (Fig. 3.2a,c), further confirming deletion of exon 2. Western blot analysis using an affinity-purified anti-LBH antibody showed that LBH protein levels in heterozygous \( Lbh^{+/\Delta2} \) embryos were reduced to approximately 50% of endogenous WT LBH expression levels (Fig. 3.2d). Furthermore, no LBH protein
expression was detected in homozygous $Lbh^{\Delta 2/\Delta 2}$ mutant embryos (Fig 3.2d), consistent with the lack of functional protein translated from $Lbh^{\Delta 2}$ transcripts.

### 3.5 Normal Mendelian Ratio, Gross Morphology and Heart Development in $Lbh^{\Delta 2/\Delta 2}$ Embryos

Despite the absence of structural LBH protein, $Lbh^{\Delta 2/\Delta 2}$ embryos at different stages of gestation displayed no apparent abnormalities and were morphologically indistinguishable from $Lbh^{+/\Delta 2}$ and $Lbh^{+/+}$ littermates (Fig. 3.3a; data not shown), indicating that LBH is not required for embryonic development. Moreover, offspring from heterozygous $Lbh^{+/\Delta 2}$ intercrosses were born with a normal Mendelian ratio of all genotypes (Table 3.1), and homozygous $Lbh^{\Delta 2/\Delta 2}$ mutant mice were viable, exhibiting no visible physical abnormalities at birth, or abnormal postnatal growth (data not shown).

Gross morphological analysis of hearts from $Lbh^{+/+}$ and $Lbh^{\Delta 2/\Delta 2}$ mice at different embryonic and postnatal stages did not reveal any apparent morphological cardiovascular defects in $Lbh^{\Delta 2/\Delta 2}$ mutant mice (Fig. 3.3b; data not shown), despite their lack of nuclear LBH protein expression in cardiomyocytes.

**Figure 3.3**: The external phenotype of homozygous $Lbh^{\Delta 2/\Delta 2}$ and heterozygous $Lbh^{+/\Delta 2}$ embryos resembles that of wild type $Lbh^{+/+}$ embryos. Representative images of embryos at E14.5 of gestation are shown.
This finding was perhaps surprising, given that transgenic overexpression of LBH in the heart resulted in multiple cardiovascular defects (Briegel et al., 2005), although we presently cannot rule out potential defects in cardiomyocyte function, which may manifest itself under stress or with late age.

### 3.6 $Lbh^{\Delta2/\Delta2}$ Females Display Defects in Mammary Gland Development

$Lbh^{\Delta2/\Delta2}$ females displayed noticeable defects in mammary gland development (Fig. 3.4a,b), which occurs primarily after birth (Hennighausen and Robinson 1998, Watson and Khaled 2008). At the onset of puberty (4 weeks of age), mammary fat pads in $Lbh^{\Delta2/\Delta2}$ females contained a rudimentary mammary epithelial tree indistinguishable from WT glands, indicating that the mammary gland anlagen had formed normally during fetal development in the absence of LBH protein. In contrast, the growth and expansion of mammary epithelium into the fat pad during puberty (6 weeks of age) was markedly reduced in $Lbh$-deficient mice as compared to age-matched WT littermates despite the presence
of prominent terminal end buds (TEBs) at the tip of the ducts (Fig. 3.4a), which drive ductal epithelial elongation at this stage in mammary gland development (Hennighausen and Robinson 1998, Watson and Khaled 2008). At 10 weeks of age the mammary glands of \( Lbh^{\Delta2/\Delta2} \) females again resembled those of WT (Fig. 3.4a), suggesting that pubertal mammary gland outgrowth occurred with delayed kinetics but eventually catches up in mature virgin glands.

Additional defects in mammary gland morphogenesis were observed in parous homozygous \( Lbh \) mutant mice (Fig. 3.4b). The alveolar compartment in pregnant, lactating and involuting \( Lbh^{\Delta2/\Delta2} \) females was markedly reduced in size as compared to stage-matched WT glands (Fig. 3.4b), suggesting that lobulo-alveolar development was perturbed in these mice. Moreover, pups from \( Lbh^{\Delta2/\Delta2} \) mutant females were significantly less viable independent of their genotypes than pups from \( Lbh^{+/+} \) and \( Lbh^{+/\Delta2} \) females, which may indicate insufficient milk provision by these mothers due to attenuated differentiation of lobuli into secretory alveoli. Typically, \( Lbh^{\Delta2/\Delta2} \) mutant females had an average litter size of

**Figure 3.4:** Postnatal mammary gland defects in \( Lbh \) null mutant animals. (a) Carmine red whole mount analysis of virgin mammary glands of WT \( Lbh^{+/+} \) and homozygous \( Lbh^{\Delta2/\Delta2} \) mutant mice at 4 weeks/wk (onset of puberty), 6 wk (puberty), and 10 wk (maturity) of age. Representative whole mounts of \( n \geq 4 \) female mice per genotype and developmental stage analyzed are shown. Asterisks denote the presence of rudimentary mammary epithelial trees at 4 wk of age and arrows the extent of mammary epithelial outgrowth into the fat pad relative to the lymph node at later developmental stages. Scale bar indicates 1 mm. (b) Whole mount analysis of parous mammary glands from WT \( Lbh^{+/+} \) and homozygous \( Lbh^{\Delta2/\Delta2} \) mice at day 14 of pregnancy, day 12 of lactation, and day 4 of involution. Representative whole mounts of \( n \geq 2 \) mice per genotype and stage analyzed are shown. Scale bar indicates 1 mm. Higher magnifications of whole mounts show a reduction in the size of the alveolar compartments in post-partum \( Lbh^{\Delta2/\Delta2} \) glands, indicative of defects in alveologenesis. Scale bar indicates 90 \( \mu m \). (c) Immunohistochemical analysis of paraffin sections from 8 wk-old mammary glands from WT \( Lbh^{+/+} \) and homozygous \( Lbh^{\Delta2/\Delta2} \) mice using affinity-purified LBH antibody (α-LBH) shows LBH expression in basal epithelium and stroma of \( Lbh^{+/+} \) mammary glands and the absence of LBH protein in \( Lbh^{\Delta2/\Delta2} \) mutant glands.
4.6±0.5 pups at the time of weaning (3 weeks of age), whereas $Lbh^{+/\Delta 2}$ control females had 7.6±0.7 pups (n=12 litters per group; Student’s t-test; p=0.001537). The observed delays in mammary gland development were not due to an overall reduced growth rate of $Lbh$-deficient mice, as $Lbh^{\Delta 2/\Delta 2}$ mice exhibited similar body weights as their age-matched WT and heterozygous littermates (data not
shown). Immunohistochemical analysis of mammary gland sections from WT and $Lbh^{\Delta 2/\Delta 2}$ females further confirmed that no detectable LBH protein was expressed in mammary gland tissues of homozygous $Lbh^{\Delta 2/\Delta 2}$ mice (Fig. 3.4c).

3.7 Conclusions

Collectively, our results demonstrate that the $Lbh$ mutant allele we have generated is a functional conditional null allele. Initial analysis of this mouse model revealed a specific requirement of LBH for postnatal mammary gland development, whereas it is not essential for proper mammary bud formation during embryogenesis. Thus, mice with the conditional $Lbh^{\text{loxP}}$ allele will be a valuable tool to delineate the multiple functions of $LBH$ during postnatal tissue development, homeostasis, and disease, as well as to decipher the genetic interactions of LBH with important signaling pathways, such as WNT. In the next chapter (Chapter 4) we explore in great depth, the mammary gland phenotype that was brief here.
In Vivo Role of LBH in Mammopoiesis

Chapter 4: The WNT target transcription cofactor LBH is an essential regulator of the basal epithelial cell lineage and stemness in the mammary gland
4.1 Background

Stem cells are important for normal organ development and adult tissue homeostasis. Their potential use in regenerative medicine and crucial involvement in a number of deleterious human diseases, most notably cancer, is receiving increasing attention (Visvader 2011, Green and Lee 2013). However, the molecular mechanisms underlying the maintenance, self-renewal and differentiation of adult stem cells remain poorly understood.

The mammary gland (MG) represents an ideal model system to study postnatal stem cell regulation because it is one of few organs that primarily develops after birth (Hennighausen and Robinson 2005, Watson and Khaled 2008) and possesses a tremendous regenerative capacity throughout most of adult life (Kordon and Smith 1998). The MG is organized into lobular and ductal structures that are nestled within the mammary fat pad and are responsible for producing and transporting milk, respectively, during pregnancy. Both the ducts and lobuli are composed of an inner layer of luminal epithelial cells surrounded by an outer layer of contractile basal myoepithelial cells (ER-, Keratin5+/6+).

The hierarchy of cell lineage specification within the MG remains incompletely characterized and much debated (Visvader 2009, Van Keymeulen, Rocha et al. 2011). The identification of mammary epithelial cells (MECs) that can reconstitute an entire functional MG at the single cell level upon transplantation (Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006) has suggested that the two mature epithelial cell lineages originate from a small population of pluripotent mammary stem cells (MaSCs). It is thought that these
MaSC then give rise to a common ‘bi-potential’ progenitor from which more committed luminal and myoepithelial progenitors arise (Asselin-Labat 2007, Sleeman, Kendrick et al. 2007, Visvader 2009). Enrichment of these MaSCs was achieved using FACS analysis and cell surface markers CD29/49f and CD24 to specifically isolate a subset of basal MEC that are CD29/49f\textsuperscript{hi}CD24\textsuperscript{+}/mod\textsuperscript{Sca1}/low as well as steroid hormone receptor negative (Asselin-Labat, Shackleton et al. 2006, Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006, Sleeman, Kendrick et al. 2007). Other in vitro studies using primary human MECs have suggested that a dynamic equilibrium of differentiated vs. undifferentiated cells may exist, in which differentiated epithelial cells can dedifferentiate into MaSC-like cells (Chaffer, Brueckmann et al. 2011) though the molecular mechanisms governing this plasticity, MaSC homeostasis and lineage specification remain ill defined. A better understanding of normal mammopoiesis is crucial as it enhances the understanding of how different breast cancer sub-types form during tumorigenesis. Thus, delineating the biological underpinnings of MG biology is an intense area of investigation that ultimately may yield new cancer therapies.

Our previous work has identified a novel key WNT-controlled transcriptional regulator, LBH (Limb-Bud and Heart). (Briegel and Joyner 2001, Briegel, Baldwin et al. 2005, Rieger, Sims et al. 2010). Initially, \textit{Lbh} was identified as a mouse gene with a unique expression pattern in the embryonic limb bud and heart, while other studies have shown \textit{Lbh} orthologs in other vertebrate species (Xenopus; Chicken) to be specifically expressed in pluripotent stem cells before
and at gastrulation (Alev, Wu et al., Paris, Osborne et al. 1988, Paris and Philippe 1990). Lbh encodes a small structurally disordered 12.3 kDA protein with transcriptional co-activator and co-repressor functions (Al-Ali, Rieger et al., Briegel and Joyner 2001, Briegel, Baldwin et al. 2005). Experimental Lbh gain-of-function in embryonic heart and bone development in vivo, as well as in vitro by LBH overexpression in the murine breast epithelial cell line HC11 results in delayed differentiation and increased proliferation of progenitor cells (Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009, Rieger, Sims et al. 2010). RNA in situ hybridization analysis demonstrated that Lbh is normally expressed in the basal myoepithelial layer of the MG which has been shown to house the multi-potent MaSC compartment of cells (Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006, Rieger, Sims et al. 2010, Van Keymeulen, Rocha et al. 2011). Lbh loss-of-function studies using ubiquitous Lbh knockout mice demonstrate pubertal MG outgrowth defects (Lindley and Briegel 2013). We have also shown that LBH correlates with WNT pathway hyperactivation in breast cancer, where it is specifically overexpressed in aggressive basal-like triple negative breast cancers (TNBC) (Rieger, Sims et al. 2010), which are enriched in breast cancer stem cells (BCSCs) (Honeth, Bendahl et al. 2008).

2002) and mutations in p63 are implicated in a broad spectrum of human syndromes with deficiencies in epithelial homeostasis (Rinne, Bolat et al. 2009). Two p63 isoforms are produced from differential promoter usage: ΔNp63 and TAp63 (Yang, Kaghad et al. 1998) with each isoform having three different C-terminal variants due to differential mRNA splicing (α, β, and γ) (Yang, Kaghad et al. 1998). In the MG, ΔNp63 is expressed in the stem cell compartment and numerous functional studies provide evidence that the ΔNp63 isoform is responsible for self-renewal of stem cells. In contrast, TAp63 is expressed in more differentiated luminal cells (Yang, Kaghad et al. 1998, Nylander, Vojtesek et al. 2002, Li, Li et al. 2006, Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010). Studies in breast epithelial cells indicate that ΔNp63 maintains a basal/MaSC epithelial phenotype and that downmodulation of ΔNp63 through the action of both Hedgehog and Notch signaling is an important step in the differentiation of basal/MaSCs into luminal progenitors (Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010). The molecular mechanisms underlying ΔNp63 function involve transcriptional regulation of basal keratins (Keratin 5), cell adhesion genes (different integrins) and cellular quiescence (Yalcin-Ozuysal, Fiche et al. 2010).

To gain insight into the roles of LBH in breast epithelial development and oncogenesis, we have explored its normal in vivo function in mammopoiesis by generating a conditional Lbh knockout mouse model. We show that in mammary epithelium of wild-type mice, LBH is predominantly expressed in the MaSC-enriched CD29hiCD24lo basal cell subpopulation. Lbh deficiency leads to a
pronounced delay in pubertal MG outgrowth, as well as increased luminal differentiation at the expense of basal myoepithelial differentiation. These defects could be traced to a severe reduction in the number and self-renewal/differentiation potential of MaSCs. Mechanistically, we identify LBH as a positive upstream regulator of epithelial stem cell transcription factor ∆Np63, promoting a basal stem cell phenotype and repressing expression of luminal cell-specific genes. Taken together, these studies establish LBH as a novel regulator of MaSC maintenance and basal lineage specification, which raises important implications for its potential role in the pathogenesis of breast cancer.

4.2 Differential expression of LBH in distinct mammary epithelial subpopulations

We have previously shown that during postnatal mouse MG development, Lbh mRNA is expressed with a dynamic pattern predominantly in the outer basal-myoeipithelial cell layer and stromal cells at virgin stages, and in the expanding lobulo-alveolar compartment of pregnant glands, whereas it is virtually absent in terminally differentiated lactating glands (Rieger, Sims et al. 2010). Moreover, ubiquitous Lbh gene ablation in mice resulted in multiple postnatal MG defects (Lindley and Briegel 2013). As the mammary epithelial compartments expressing Lhb have been shown to be enriched in stem/progenitor cells (Asselin-Labat, Vaillant et al., Shackleton, Vaillant et al. 2006, Sleeman, Kendrick et al. 2007) and the MG defects in Lbh null mice coincide with stages of active MaSC
expansion and differentiation (Lindley and Briegel 2013), we sought to further explore the role of LBH in mammopoiesis.

We first determined the localization of LBH protein expression in mammary epithelial structures by performing Immunohistochemical (IHC) analysis using affinity-purified LBH anti-body (see Methods) on paraffin-embedded tissue sections from 8-week (wk) old virgin MGs. Intense nuclear staining of LBH protein was observed in a subset of cells within the basal myoepithelial layer and in stromal cells (Fig 4.1A). In contrast, LBH was not or only weakly expressed in luminal cells of the inner epithelial layer of the ducts. Comparative analysis of cell lineage marker expression on serial MG sections showed co-localization of LBH with basal epithelial lineage marker keratin 5 (K5), whereas LBH expression was mutually exclusive with luminal epithelial lineage markers, keratin 8 (K8) and Estrogen Receptor alpha (ERα) (Fig 4.1A). Additionally, LBH was expressed in cap cells and individual body cells of the

Figure 4.1: LBH is expressed predominantly in basal epithelium and the MaSC-containing CD29loCD24hi subpopulation of the mammary gland. (A) Immunohistochemical (IHC) analysis of paraffin-embedded serial mammary gland tissue sections from 8 wk-old virgin female mice showing co-localization of LBH protein with basal Keratin 5 (K5) in the basal epithelial layer, and its exclusion from the luminal epithelial layer, which expresses Keratin 8 (K8) and Estrogen Receptor alpha (ERα). Close-up views on individual areas (black boxes) are shown. Red arrows mark basal and red asterisks stromal cells. (B) IHC staining of formalin-fixed paraffin-embedded human breast tissue sections with LBH-specific antibody (brown). (C) Fluorescence activated cell sorting (FACS) was used to segregate freshly isolated lineage-negative (Lin: CD31, CD45-, TER119-) mammary epithelial cells from 8 wk-old virgin female mice into CD29loCD24hi luminal and CD29hiCD24+ basal subpopulations for subsequent RNA extraction and qPCR analysis (see C). A representative FACS dot plot is shown. (D) Quantitative RT-PCR (qPCR) analysis of Lbh and epithelial lineage marker expression in CD29loCD24hi luminal and CD29hiCD24+ basal cells relative to Gapdh. Data represent mean±SEM of three independent experiments. Statistically significant differences of p<0.05 (t-test) were observed between the two groups for each of the seven genes shown.
terminal end buds (TEBs), which are transient structures at the leading edge of the ducts and are enriched in activated MaSCs driving ductal elongation (Bai and Rohrschneider 2010) (Fig. 4.3B; data not shown). Furthermore, in human breast tissues, expression of LBH in both the ducts and alveoli was restricted to cells
within the baso-myoepithelial layer and to rare supra-basal cells, which have also been shown to express stem cell markers (Van Keymeulen, Rocha et al.) (Fig. 4.1B). These results indicate that in both, murine and human MGs, LBH is specifically expressed in a subset of epithelial cells within the basal cell compartment, while it is excluded from more differentiated luminal epithelial cells.

To identify the epithelial subpopulations in which LBH was expressed, we examined Lbh mRNA expression in fluorescence-activated cell sorting (FACS) isolated mammary epithelial cell (MEC) populations using quantitative reverse transcriptase-polymerase chain reaction (qPCR) analysis. Freshly isolated cells from virgin MGs at 8 wk of age were sorted into luminal Lin-CD29<sup>lo</sup>CD24<sup>hi</sup> and basal Lin-CD29<sup>hi</sup>CD24<sup>+</sup> (herein referred to as CD29<sup>lo</sup>CD24<sup>hi</sup> or CD29<sup>hi</sup>CD24<sup>+</sup>, respectively) epithelial cell populations (Fig. 4.1C). The basal CD29<sup>hi</sup>CD24<sup>+</sup> epithelial cell fraction is enriched in multipotent MaSCs but also comprises mature myoepithelial cells (Shackleton, Vaillant et al. 2006). The purity of cell subpopulations was evaluated by determining expression of luminal (K8) and basal (K5) markers (Fig. 4.1D). Additionally, Lbh expression was compared to expression of luminal differentiation marker ER<sub>α</sub>, the luminal cell-specific TAp63 and basal stem cell-specific ΔNp63 isoforms of epithelial transcription factor p63 (Nylander, Vojtesek et al. 2002, Li, Singh et al. 2008), as well as Axin 2, which like LBH is a direct WNT target gene (Jho, Zhang et al. 2002, Rieger, Sims et al. 2010), known to be expressed in mammary cells with stem/progenitor activity (Zeng and Nusse 2010). As anticipated, K8, ER<sub>α</sub> and TAp63 expression was highest in the CD29<sup>lo</sup>CD24<sup>hi</sup> luminal subpopulation,
whereas expression of K5 and ∆Np63 was restricted to the MaSC-enriched CD29\textsuperscript{hi}CD24\textsuperscript{+} basal subpopulation. Notably, \textit{Lbh} was predominantly expressed in the basal K5\textsuperscript{+}∆Np63\textsuperscript{+}ER\textsubscript{α} MaSC fraction rather than in the K8\textsuperscript{+}TAp63\textsuperscript{+}ER\textsubscript{α} luminal cell fraction (~6 fold; p<0.01) (Fig. 4.1D; Fig. 4.3A). The \textit{Lbh} expression profile was most similar to that of Axin 2, which was also significantly enriched (>3 fold; p<0.01) in the CD29\textsuperscript{hi}CD24\textsuperscript{+} basal stem cell fraction (Fig 4.1D).

To further evaluate whether LBH is associated with MaSCs, we investigated \textit{Lbh} expression in \textit{Lgr5-GFP-CreER\textsuperscript{T2}} reporter mice (Barker, van Es et al. 2007). Lgr5, a direct WNT target gene and marker of epithelial stem cells in different tissues (Barker, van Es et al. 2007), has been shown to be enriched in the CD29\textsuperscript{hi}CD24\textsuperscript{+} MaSC population (Stingl, Eirew et al. 2006, Van Keymeulen, Rocha et al. 2011). Mammary epithelial cells from \textit{Lgr5-GFP-CreER\textsuperscript{T2}} mice, which express GFP from the endogenous Lgr5 promoter (Barker, van Es et al. 2007), were sorted into GFP-positive (GFP\textsuperscript{+}) and GFP-negative/low (GFP\textsuperscript{−}) cells in combination with a CD24-specific antibody, which was used to distinguish between CD24\textsuperscript{hi} luminal and CD24\textsuperscript{+} basal/MaSC subpopulations using FACS analysis (Fig. 4.2A). As predicted, strongly GFP\textsuperscript{+} cells clustered in the CD24\textsuperscript{+} MaSC-enriched fraction, which was characterized by basal K5 expression. Conversely, GFP\textsuperscript{−} cells clustered within the CD24\textsuperscript{hi} luminal subpopulation, which was luminal marker K8-positive (Fig. 4.2A, D). To verify that the GFP\textsuperscript{+}CD24\textsuperscript{+} population contained cells with increased stem cell activity, the two epithelial subpopulations were collected and plated as single cell suspensions in non-
adherent mammosphere cultures. In this in vitro assay, only cells with unipotent and multipotent differentiation potential can self-renew and form spheres (Dontu, Abdallah et al. 2003). Sphere formation was significantly higher (>3 fold; p<0.01) in the GFP⁺CD24⁺ cell fraction than in the GFP⁺CD24⁺i fraction of Lgr5-GFP-CreER_T2 MGs (Fig. 4.1B), consistent with the idea that Lgr5-GFP marks

![Image of flow cytometry and mammosphere culture](image)

Figure 4.2: LBH expression is restricted to GFP+ stem cells in Lgr5-GFP-CreER_T2 mice. (A) To further demonstrate that Lbh is specifically enriched in MaSCs that reside within the CD29⁺CD24⁺ basal subpopulation, we next sorted MGs from Lgr5-GFP-CreER_T2 mice. Endogenous GFP was used in combination with CD24-PE (as well as lineage markers see methods) to distinguish between GFP⁺CD24⁺ (luminal) and GFP⁺CD24⁺ (basal/MaSC) subpopulations. (B) Lgr5-GFP-CreER_T2 sorted cells were then placed in mammosphere culture for 14 days showing a significant increase in the sphere forming capacity of GFP⁺CD24⁺ as compared to GFP⁺CD24⁺i cells (>3 fold; p<0.01). (C-D) QPCR analysis of sorted cells showed enrichment of Lbh, K5 and Axin2 specifically in the GFP⁺CD24⁺ MaSC subpopulation (~9 fold, 27 fold and 3 fold respectively; p<0.01), while luminal K8 was expressed in the GFP⁺CD24⁺i cells (~4.5 fold; **p<0.01).
stem/progenitor cells. Expression analysis revealed that *Lbh*, similar to *Axin2*, which was used as a control, was expressed with highest abundance (9 fold, and >3 fold, respectively; \(p<0.01\)) in the GFP\(^+\)CD24\(^+\) MaSC population as compared to the GFP\(^-\)CD24\(^{hi}\) luminal fraction (Fig. 4.2C). Collectively, these data suggest that *Lbh* is specifically expressed in a rare population of stem-like cells in the basal epithelial compartment, raising the notion that LBH may play a role in MaSC biology.

4.3 Loss of *Lbh* results in delayed pubertal mammary gland outgrowth

We next used a loss-of-function strategy to study the *in vivo* role of LBH in the mammary epithelial development. 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 recombination results in deletion of exon 2, giving rise to a severely truncated and non-functional LBH protein of 8 amino acids (Lindley and Briegel 2013). Conditional *Lbh\(^{loxP}\)* mice were crossed with transgenic mice expressing Cre recombinase under the control of the keratin 14 (K14) promoter, which is active in basal epithelia of the skin and MG, but also transiently in luminal mammary epithelial cells at pre-pubertal stages (Van Keymeulen, Rocha et al.). To confirm that the K14 promoter targets Cre-mediated *Lbh* gene inactivation to specific mammary epithelial cell lineages, basal, luminal and stromal cells were isolated from *K14-Cre; Lbh\(^{loxPloxP}\)* mice using FACS. Evaluation of *Lbh* expression by qPCR detected no functional *Lbh* mRNA
transcripts in CD29\textsuperscript{hi}CD24\textsuperscript{+} basal cells and a severe reduction of the low-level expression of \textit{Lbh} in CD29\textsuperscript{lo}CD24\textsuperscript{hi} luminal cells of \textit{Lbh} mutant MGs as compared to the respective cell populations of glands from wild type (WT) littermates (Fig. 4.3A). Epithelial-specific ablation of LBH expression in \textit{K14Cre;Lbh}\textsuperscript{loxP/loxP} MGs was further confirmed by IHC protein analysis of 8-wk-old MG sections, which, together with the qPCR analysis, revealed that LBH was still expressed in individual stromal cells (Fig. 4.3A, B), which lack K14 promoter activity.

Next, we examined the consequences of epithelial-specific \textit{Lbh} deletion during puberty, as this crucial stage in postnatal MG development is characterized by the massive expansion and differentiation of long-term populating MaSCs (Watson and Khaled 2008). Since ubiquitous \textit{Lbh} null mice, which we obtained after breeding of \textit{Lbh}\textsuperscript{loxP} mice with a \textit{ROSA26-Cre (R26-Cre)} deleter mouse strain, are viable (Lindley and Briegel 2013), we also included these mice in our analysis. At the beginning of puberty (3-4 weeks) (Watson and Khaled 2008), when the TEBs at the leading edge of the glands have just reached the lymph node, \textit{Lbh}-deficient MGs from both Cre lines were indistinguishable from WT glands (Lindley and Briegel 2013) (data not shown). However, at 6 weeks of age, when puberty is ongoing and the mammary epithelium had started to invade the fat pad in WT mice, there was a severe reduction in ductal elongation in both \textit{K14Cre;Lbh}\textsuperscript{loxP/loxP} and \textit{R26Cre;Lbh}\textsuperscript{loxP/loxP} mutant mice relative to WT glands (~60\% and 50\%, respectively; \(p<0.01\)), as quantified by measuring the distance of TEB migration from the center of the lymph node (Fig. 4.2C, D; Fig 4.4B,C). The impairment in ductal elongation
persisted at 8 wks of age in mutant mice of both lines (50% and 25% growth reduction, respectively, p<0.01, p=0.1). MG outgrowth eventually caught up in older virgin mice (10-12 weeks) (Fig. 4.3B, C; Fig 4.4B, C; data not shown), suggesting that loss of LBH specifically delayed pubertal mammary gland outgrowth.

To assess whether the LBH-dependent failure in epithelial growth was a result of decreased proliferation, sections from MGs at 8 wks of age were stained for Ki67, a marker for proliferation (Fig. 4.3E, F; data not shown). Puberty-induced ductal elongation is driven by the massive proliferation of stem/progenitor cells in the terminal end buds (TEB) that invade the mammary fat pad (Watson and Khaled 2008, Bai and Rohrschneider 2010). Accordingly, in WT the majority of Ki67+ cells (>60%) were detected in the TEBs, whereas the ducts contained <6% of Ki67+ cells (Fig. 4.3F). In K14Cre;LbhloxP/loxP mutant

**Figure 4.3:** Ablation of Lbh results in delayed pubertal mammary gland outgrowth and reduced levels of TEB proliferation in Keratin14-Cre;LbhloxP/loxP mice. (A) QPCR analysis of FACS-sorted luminal (CD29loCD24hi), basal (CD29hiCD24+), and stromal (CD29+CD24-) cell-enriched populations demonstrating specific loss of Lbh expression in basal and luminal epithelial cells of Keratin14 (K14)-Cre;LbhloxP/loxP mice but not in stromal cells as compared to wild type (WT). Values were normalized to Gapdh and represent means ± SEM (n=3 mice per genotype). (B) IHC analysis of paraffin sections from 8 wk-old mammary glands from K14-Cre;LbhloxP/loxP and respective WT littermates shows the absence of LBH protein expression in the epithelium of Lbh-mutant glands. Basal cell-specific expression of LBH in WT (red arrows) and stromal cell-specific expression in WT and mutants (red asterisks) is indicated. TEB = terminal end buds. (C) Representative whole mounts of mammary glands from K14-Cre;LbhloxP/loxP virgin mice at 6 wk and 8 wk of age, scale bar= 2 mm. (D) Quantification of epithelial outgrowth into the mammary fat pad, as measured in mm from the center of the lymph node. Values represent means ± SD (n=3 animals per genotype). **p<0.01, Student t-test. (E) Representative images of IHC for Ki67 (brown) shows reduced proliferating rates in TEBs of K14-Cre;LbhloxP/loxP mice, as quantified in F. (F) The percentage (%) of Ki67-positive cells in ducts and TEBs was calculated from >3 different random sections per gland. Values represent means ± SD (n=3 mice per genotype). **p<0.01, Student t-test.
Figure 4.4: Ablation of Lbh results in delayed pubertal mammary gland outgrowth in Rosa26-Cre;Lbh^{loxP/loxP} mice. (A) QPCR analysis of FACS-sorted luminal (CD29<sup>lo</sup>CD24<sup>hi</sup>), basal (CD29<sup>hi</sup>CD24<sup>+</sup>), and stromal (CD29<sup>+</sup>CD24<sup>-</sup>) cell-enriched populations demonstrating ubiquitous loss of Lbh expression in all cells of Rosa26 (R26)-Cre;Lbh^{loxP/loxP} mice as compared to wild type (WT). Values were normalized to Gapdh and represent means ± SEM (n=3 mice per genotype). (B) IHC analysis of paraffin sections from 8 wk-old mammary glands from R26-Cre;Lbh^{oxP/loxP} and respective WT littermates shows the absence of LBH protein expression in the epithelium of Lbh mutant glands. Basal cell-specific expression of LBH in WT (red arrows) and stromal cell-specific expression in WT and mutants (red asterisks) is indicated. TEB = terminal end buds. (C) Representative whole mounts of mammary glands from R26-Cre;Lbh^{oxP/loxP} virgin mice at 6 wk and 8 wk of age, scale bar=2 mm. (D) Quantification of epithelial outgrowth into the mammary fat pad, as measured in mm from the center of the lymph node. Values represent means ± SD (n=3 animals per genotype). **p<0.01, at 6 weeks, Student t-test.
glands, the number of Ki67+ cells in the ducts was the same as in WT glands, however, the number of Ki67+ cells in the TEBs was significantly reduced (>25%; p<0.01) (Fig 4.3E, F). Moreover, immunostaining of sections for activated caspase 3 detected <1% of cells positive in MGs of both genotypes (data not shown), confirming that the observed outgrowth defect is not a consequence of increased apoptosis, but results from impaired progenitor cell expansion in the TEBs.

4.4 Loss of *Lbh* results in abnormal mammary epithelial cell morphology and lineage differentiation

Histological analysis revealed that overall the bi-layer structure of the MGs from *K14-Cre;Lbh<sup>loxP/loxP</sup>* and *R26Cre;Lbh<sup>loxP/loxP</sup>* mutant mice was intact, with an inner layer of luminal cells surrounded by an outer layer of baso-myoepithelial cells similar to WT (Fig 4.5A; Fig. 4.7A). However, whereas in WT glands, basal cells had a typical flat and 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 MGs was abnormally thickened, disorganized, and the nuclei of luminal cells were enlarged in size as compared to age-matched WT glands (Fig 4.5A; Fig. 4.7A), indicating that both the basal and luminal compartments were affected by loss of *Lbh*. To assess whether loss of *Lbh* perturbed basal-luminal cell specification and/or differentiation, IHC analysis of lineage-specific marker expression was performed
While expression of luminal cytokeratin K8 was virtually unchanged in both ducts and TEBs of Lbh-deficient MGs, indicating normal luminal lineage specification, expression of the basal cytokeratin K5 was visibly reduced in the basal epithelial cell layer of LBH mutant glands as compared to WT (Fig. 4.5B; Fig. 4.7B). Most notably, K14-Cre;LbhloxP/loxP MGs completely lacked the characteristic expression of stem cell transcription factor ΔNp63 in basal cells, whereas stromal expression of this protein was unaffected by epithelial-specific loss of Lbh (Fig. 4.5C). In contrast, the luminal cell-specific isoform of p63, TAp63, was abnormally overexpressed in the luminal epithelial cell layer of K14-Cre;LbhloxP/loxP mutant relative to control WT glands (Fig. 4.5C). Furthermore, hormone receptor ERα, was expressed in far greater numbers of luminal cells (60%) and with increased immunostaining intensity in Lbh-deficient MGs (in both K14-Cre and R26-Cre deleter strains) compared to WT glands, which expressed ERα in ~28% of luminal cells (Fig. 4.6A, B; Fig. 4.7C). As ERα signaling plays an important role in pubertal ductal elongation, as well as mammary epithelial cell proliferation and differentiation (Mallepell, Krust et al. 2006), we further addressed, whether ERα was transcriptionally active in Lbh knockout mice. Serial MG sections from K14-Cre;LbhloxP/loxP, R26Cre;LbhloxP/loxP mutant mice, and respective WT control littermates were stained with antibodies specific to progesterone receptor (PR). PR is a direct ERα target gene and accordingly, PR levels and the number of PR-expressing cells were markedly increased in the luminal cell layer of Lbh-deficient mice, mirroring the changes in ERα expression (Fig. 4.6A, B; Fig. 4.7C). Serum estradiol concentrations were
unchanged in K14-Cre;Lbh\textsuperscript{loxP/loxP} mice as compared to WT mice (Fig. 4.6C), indicating that the aberrant luminal ER\(\alpha\) overexpression and increased activity in Lbh knockout mice was not due to changes in systemic hormone levels.

To quantify the LBH-dependent changes in basal-luminal marker expression, we fractionated mammary cells from WT and K14-Cre;Lbh\textsuperscript{loxP/loxP} knockout mice into the different epithelial subpopulations using FACS analysis followed by qPCR expression analysis of lineage markers (Fig 4.8; Fig. 4.10F). Consistent with our in situ protein analysis, mRNA levels of basal K5 and stem cell markers \(\Delta Np63\) and Axin 2 were significantly reduced (by \(>60\%\) and \(~50\%\) respectively; \(p<0.01\)) in the CD29\textsuperscript{hi}CD24\textsuperscript{+} basal subpopulation. Conversely, luminal differentiation markers ER\(\alpha\) and TAp63 were profoundly upregulated (ca. 4-fold each, \(p<0.01\)) in the CD29\textsuperscript{lo}CD24\textsuperscript{hi} luminal cell fraction of K14-Cre;Lbh\textsuperscript{loxP/loxP} MGs, although luminal lineage marker K8 expression levels were only modestly increased (Fig. 4.8). Similar changes in mRNA expression levels of these luminal-basal markers were also observed in FACS-sorted basal and luminal epithelial cell populations from R26Cre;Lbh\textsuperscript{loxP/loxP} mutant glands (Fig. 4.10F). Of note, we also analyzed expression of other transcription factors known to control basal stem cell (e.g. Slug) or luminal (e.g. GATA3) cell fates and

**Figure 4.5:** Loss of Lbh alters mammary epithelial cell morphology and basal-luminal lineage differentiation in Keratin14-Cre;Lbh\textsuperscript{loxP/loxP}. (A) Hematoxylin and Eosin (H&E) analysis of wild type (WT) and K14-Cre;Lbh\textsuperscript{loxP/loxP} mutant mammary glands; red arrows indicate normal basal morphology in WT and aberrant morphology in K14-Cre;Lbh\textsuperscript{loxP/loxP} respectively. (B) IHC staining for basal Keratin 5 (K5) shows decreased expression (red arrows) in K14-Cre;Lbh\textsuperscript{loxP/loxP} mammary gland, whereas expression of luminal Keratin 8 (K8) is virtually unchanged. (C) IHC analysis of lineage-specific p63 isoform expression shows the absence of \(\Delta Np63\) expression in basal cells (red arrows) of K14-Cre;Lbh\textsuperscript{loxP/loxP} mutant glands, whereas it is still expressed in stromal cells (red asterisk). Conversely expression of the luminal p63 isoform, TAp63, is markedly upregulated in luminal cells (red arrowheads) of K14-Cre;Lbh\textsuperscript{loxP/loxP} glands.
**Figure 4.6:** Increased estrogen hormone receptor expression and activity in Keratin14-Cre;Lbh^{loxP/loxP} mice despite normal systemic Estradiol levels. (A) IHC staining of sections from K14-Cre;Lbh^{loxP/loxP} and matched WT mammary glands for luminal ERα and PR expression (brown) shows significant increases in intensity and number of luminal cells (indicated by red arrowheads) staining positive for ERα and PR in both the ducts and TEBs (as quantified in (B)). (B) The percentages (%) of ERα and PR positive cells were calculated from >3 different random sections per gland. Values represent mean ± SEM (n=3 mice per genotype). ***p<0.001, Student t-test. (C) Serum estradiol levels (in picogram/ml) were not significantly changed in K14-Cre; Lbh^{loxP/loxP} females at 8-wk-of-age relative to WT littermates. Values represent means ± SD (n=3 mice per genotype).
Figure 4.7: Ubiquitous loss of Lbh alters mammary epithelial cell morphology, basal-luminal lineage differentiation and results in increased expression of hormone receptors ERα and PR. (A) Hematoxylin and Eosin (H&E) analysis of wild type (WT) and R26-Cre; LbhloxP/loxP mutant MGs; red arrows indicate normal basal morphology in WT and aberrant morphology in R26-Cre; LbhloxP/loxP respectively. (B) IHC analysis of basal Keratin 5/6 shows decreased expression in the R26-Cre; LbhloxP/loxP MG, whereas expression of luminal Keratin 8 is virtually unchanged. (C) IHC analysis of ERα and target gene PR, shows significant increase in intensity and number of cells staining positive for ERα and PR in R26-Cre; LbhloxP/loxP as compared to WT.
differentiation (Guo, Keckesova et al., Kouros-Mehr, Slorach et al. 2006, Asselin-Labat, Sutherland et al. 2007). However, in contrast to ΔNp63, TAp63, and ERα, mRNA levels of these lineage-specific regulatory molecules did not significantly change in the respective epithelial subpopulations of K14-Cre;LbhloxP/loxP MGs (Fig. 4.8). Taken together, these data suggest that loss of Lbh specifically impairs the MaSC-containing basal epithelial compartment, as well as alters the differentiation status of luminal cells, while it does not appear to affect luminal cell specification.

**Figure 4.8:** Ablation of Lbh perturbs epithelial lineage marker expression in mammary epithelial subpopulations in Keratin14-Cre;LbhloxP/loxP mice. QPCR analysis of freshly isolated FACS sorted luminal (CD29loCD24hi) and basal (CD29hiCD24+) mammary epithelial cells from WT and K14-Cre;LbhloxP/loxP mice at 8-wk-of-age reveals a significant increase in expression levels of luminal differentiation markers Keratin 8, ERα, and TAp63 (~40% and ~400% respectively; p<0.05 and p<0.01; n=3) in luminal subpopulation of K14-Cre;LbhloxP/loxP glands. In contrast, expression levels of basal Keratin 5 (K5) and stem cell markers ΔNp63 and Axin 2 were significantly reduced in the basal subpopulation of K14-Cre;LbhloxP/loxP mice (~50% reduction; p<0.01; n=3) as compared to the respective WT mammary epithelial subpopulations. Unexpectedly, no significant changes in the expression levels of key luminal (Gata3) and basal (Slug) transcription factors were apparent upon LBH knockout. Values were normalized to Gapdh and represent the mean ± SEM (n=3).
4.5 Loss of Lbh decreases the frequency and activity of mammary stem cells

To identify the potential causes of the observed mammary outgrowth defects and abnormal basal-luminal cell differentiation in Lbh-deficient mice, we next examined the effects of LBH loss on MaSCs. FACS analysis and mammosphere assays were used to address the distribution and functionality of MaSCs in vitro (Fig. 4.9; Fig. 4.10). Analysis of primary mammary cells from 8 wk-old virgin mice from each genotype (n=4) revealed a striking reduction (40-50%; p<0.05) in the percentage of the basal CD29^hiCD24^+ population from K14-Cre;Lbh^{loxP/loxP} glands as compared to WT controls (Fig 4.9A, B). Additionally, the percentage of the luminal CD29^loCD24^hi subpopulation was slightly but significantly increased (~10%; p<0.05; Fig. 4.9A, B). Similar changes in the distribution of the CD29^hiCD24^+ and CD29^loCD24^hi epithelial subpopulations were also observe in age-matched R26-Cre;Lbh^{loxP/loxP} mice (Fig. 4.10A). As a first approximation of stem cell activity, whole mammary epithelial cell populations were isolated from K14-Cre;Lbh^{loxP/loxP}, R26-Cre;Lbh^{loxP/loxP}, and respective WT mice and seeded into non-adherent mammosphere cultures. Unsorted mammary epithelial cells from Lbh-deficient mice in either Cre-deleter background displayed a significant reduction (approximately 50%; p<0.001) in their sphere forming capacity as compared to WT (Fig 4.9C; Fig. 4.10B). Subsequent sphere assays with FACS-sorted epithelial subpopulations revealed that the decline in primary sphere formation ability of Lbh-deficient MECs was specifically due to
reduced activity of the basal MaSC population, as the CD29[^hi]CD24[^+] cell fraction from \( Lbh \)-deficient mice in both Cre-deleter strains gave rise to 80% less primary spheres than the same cell population from WT mice (Fig 4.9D; Fig. 4.10C). In contrast, no significant differences in the sphere formation capacity of luminal CD29[^lo]CD24[^hi] cells were evident between \( Lbh \) knockout and WT mice. To address, whether the self-renewal capacity of MaSCs was affected by loss of LBH, secondary sphere assays were performed after dissociation of primary spheres originating from sorted basal and luminal epithelial subpopulations of \( K14-Cre;Lbh^{loxP/loxP} \) MGs. In this assay, the CD29[^hi]CD24[^+] basal fraction from WT.

**Figure 4.9:** Loss of \( Lbh \) reduces the frequency, activity, and differentiation potential of CD29[^hi]CD24[^+] basal stem cells in \( Keratin14-Cre;Lbh^{loxP/loxP} \) mice. (A) CD24/CD29 FACS dot plots showing the distributions of different subpopulations within the Lin- (Lin: CD45/CD31/TER119) cell fraction from WT and \( K14-Cre;Lbh^{loxP/loxP} \) glands at 8-wk-of-age. (B) Histogram showing the percentages (%) of luminal CD29[^lo]CD24[^hi] and basal CD29[^hi]CD24[^+] subpopulations from WT and \( K14-Cre;Lbh^{loxP/loxP} \) glands (mean ± SD of four animals per group). *p<0.05 \( Lbh \)-deficient vs. WT mice. (C-E) Sphere assays (14 days) using single cell suspensions of freshly isolated (C) unsorted mammary cells; (D) CD24-CD29 FACS sorted primary mammary epithelial cells populations, and (E) dissociated primary spheres derived from individual luminal (CD29[^lo]CD24[^hi]) and basal (CD29[^hi]CD24[^+]) subpopulations (see D) from WT and \( K14-Cre;Lbh^{loxP/loxP} \) glands. Values represent mean ± SD (n=4 animals per group). *p<0.05; **p<0.01 \( Lbh \)-deficient mice (black bars) vs. WT (white bars). Notably, mammary cells from \( K14-Cre;Lbh^{loxP/loxP} \) glands exhibit a significantly reduced sphere formation capacity (~50%) (C), which could be attributed to both, reduced activity and self-renewal of the basal (CD29[^hi]CD24[^+]) cell fraction, as evident by the primary (D) and secondary (E) sphere assays respectively. (F) Representative immunofluorescence images of differentiation-induced primary mammospheres (see Methods) derived from the CD29[^hi]CD24[^+] MaSC populations from WT and \( K14-Cre;Lbh^{loxP/loxP} \) glands (see D) stained with antibodies for basal (K5; red) and luminal (K8; green) markers. (G) Histogram showing the percentages (%) of K5+ basal (black), K8+ luminal (white), and K5+/K8+ bi-potential progenitor cells (grey) formed from primary mammospheres after 5 days of growth under adherent differentiation conditions, as shown in (F). These percentages were derived from counting cells in 10 differentiated spheres from N=3 animals per genotype. The number of K5+ basal cells was significantly reduced (~62%; p<0.05), whereas the number of luminal K8+ cells was increased (~100%; p<0.05) in \( Lbh \)-deficient mice as compared to WT.
A

WT

K14-Cre: Lbh^{lox}P_2^{loxP}

CD29^{hi}CD24^{lo} (luminal)

CD29^{hi}CD24^{+} (basal/MaSC)

B

K14-Cre

% population

CD29^{lo} CD29^{hi} CD24^{+}

C

K14-Cre Unsorted

D

K14-Cre 1° Sorted

E

K14-Cre 2° Sorted

F

K14-Cre

WT Lbh^{lox}P_2^{loxP}

G

K14-Cre

Keratin 5+

Keratin 8+

Keratin 8+/5+

Sphere Staining Distribution
glands showed a pronounced enrichment in sphere formation as compared to the CD29loCD24hi luminal cell population (Fig. 4.9E). However, this enrichment in secondary sphere formation did not occur for CD29hiCD24+ cells derived from K14-Cre;LbhloxP/loxP MGs, which showed a significantly reduction in sphere activity (by ~50%; \( p<0.05 \)) as compared to the same MEC population from WT glands (Fig 4.9E). Collectively, these results suggest that loss of LBH adversely affects the in vitro self-renewal potential of MaSCs in the basal subpopulation, while it does not appear to alter luminal cell progenitor activity.

**Figure 4.10:** Ubiquitous loss of *Lbh* reduces the frequency, activity, and differentiation potential of CD29hiCD24+ basal stem cells in Rosa26-Cre;LbhloxP/loxP mice. (A) Histogram showing the percentages (%) of luminal CD29loCD24hi and basal CD29hiCD24+ subpopulations from WT and R26-Cre;LbhloxP/loxP glands (mean ± SD of four animals per group). **p<0.01 Lbh-deficient vs. WT mice. (B-C) Sphere assays (14 days) using single cell suspensions of freshly isolated (B) unsorted mammary cells and (C) CD24-CD29 FACS sorted primary luminal (CD29loCD24hi) and basal (CD29hiCD24+) subpopulations from WT and R26-Cre;LbhloxP/loxP glands. Values represent mean ± SD (n=4 animals per group). **p<0.01; **p<0.01 Lbh-deficient mice (black bars) vs. WT (white bars). (D) Representative immunofluorescence images of differentiation-induced primary mammospheres (see Methods) derived from the CD29hiCD24+ MaSC populations from WT and R26-Cre;LbhloxP/loxP glands (see C) stained with antibodies for basal (K5; red) and luminal (K8; green) markers. (E) Histogram showing the percentages (%) of K5+ basal (black), K8+ luminal (white), and K5+/K8+ bi-potential progenitor cells (grey) formed from primary mammospheres after 5 days of growth under adherent differentiation conditions, as shown in (C). These percentages were derived from counting cells in 10 differentiated spheres from N=3 animals per genotype. The number of K5+ basal cells was significantly reduced (~86%; \( p<0.01 \)), whereas the number of luminal K8+ cells was increased (~150%; \( p<0.01 \)) in Lbh-deficient mice as compared to WT. (F) QPCR analysis of FACS sorted basal (CD29hiCD24+) and luminal (CD29loCD24hi) MECs reveals reduced expression of basal *Keratin 5* and stem cell marker *Axin 2* in basal MECs (~70% reduction \( p<0.01 \)). Luminal differentiation marker *Keratin 8* showed an insignificant change in transcript level while *ERα* was significantly increased (~900%; \( p<0.001 \)) in luminal MECs of R26-Cre; LbhloxP/loxP as compared to WT.
4.6 Lbh deficient mammary stem cells have an altered differentiation potential yielding an increased luminal (K8+) progeny in vitro

To further ascertain that Lbh deficiency specifically affected the functionality of MaSCs, we assessed their ability to differentiate into the different mammary epithelial lineages in vitro. Only multipotent MaSCs in the basal mammary epithelial compartment can give rise to both the basal-myoeptheial
and luminal cell lineages, whereas stem/progenitor cells in the luminal compartment are unipotent and only capable to contribute to the luminal cell lineage (Van Keymeulen, Rocha et al.). Therefore, the CD29^{hi}CD24^{+} basal cell populations from $K14$-Cre;$Lbh^{loxP/loxP}$, $R26$Cre;$Lbh^{loxP/loxP}$ and respective WT mice were isolated by FACS and, after growth in mammosphere cultures for 14 days to enrich for stem cells, individual primary spheres were platted onto adherent collagen-coated culture slides to induce differentiation by (see Methods 2.2.1) (Pei, Bai et al. 2009). Spheres were maintained under differentiation conditions for 6 days, followed by co-IF staining of cells for K5 and K8 to assess basal and luminal cell differentiation (Fig. 4.9F; Fig. 4.10E). Under these conditions, spheres derived from WT CD29^{hi}CD24^{+} MaSC fractions gave rise to a majority of 76-79% of K5+/K8+ double positive cells, which likely represent bi-potential progenitor-like cells, 6–7% of K5+ single-positive basal myoepithelial cells and 15–16% of K8+ single-positive luminal cells (Fig. 4.9F,G; Fig. 4.10D,E). In contrast, spheres from $K14$-Cre;$Lbh^{loxP/loxP}$ and $R26$Cre;$Lbh^{loxP/loxP}$ CD29^{hi}CD24^{+} MaSC fractions gave rise to significantly reduced numbers of K5+/K8+ double positive progenitors (65 or 57% respectively) and showed a sharp decline in mature K5+ basal myoepithelial cells (from 6.1 to 2.3%; p<0.05; or from 7.3 to 1%; p<0.01 respectively) (Fig. 4.9F,G; Fig. 4.10D,E). Conversely, the number of differentiated K8+ luminal cells was significantly increased as compared to WT spheres (from 15 to 33%; p<0.05; or from 16.3 to 42.5%; p<0.01 respectively) (Fig. 4.9F,G; Fig. 4.10D,E). These data indicate that $Lbh$-deficient CD29^{hi}CD24^{+} basal subpopulations have reduced multipotency and are
shifted in their differentiation potential towards a more luminal, terminally
differentiated cellular phenotype at the expense of baso-myoeptithelial cell
differentiation, thus, providing a possible explanation for the observed shift from
basal to more luminal characteristics of Lbh-deficient MGs in vivo.

4.7 LBH promotes ‘stemness’ of mammary epithelial cells, which is
accompanied by induction of ∆Np63 and repression of ERα expression

Our in vivo and ex vivo studies thus far suggest that LBH is required to
maintain stem cells and the basal epithelial cell lineage, while it represses
luminal epithelial differentiation. The specific and drastic changes in the
expression levels and patterns of p63 isoforms and ERα, with basal ∆Np63 being
downregulated and luminal TAp63 and ERα being upregulated in Lbh-deficient
MGs (Fig. 4.5-4.8), led us to hypothesize that these lineage-specific transcription
factors might play a role in LBH-dependent stem cell regulation and cell
differentiation processes. To test this possibility, we examined regulation of p63
and ERα by LBH in MEC culture systems, in which Lbh was either depleted or
overexpressed. RNAi was used to efficiently deplete LBH expression in the two
normal-derived human MEC lines MCF10A and 226L, which express
endogenous LBH at low or high levels respectively (Fig. 4.11A). Of note, both of
these cell lines exhibit basal characteristics are ERα–low/negative. Conversely,
LBH was exogenously expressed in the mouse MEC line HC11 (Fig. 4.11D)
(Rieger, Sims et al. 2010), which is one of few established normal mammary
epithelial lines that endogenously express ERα and display luminal characteristics (Faulds, Olsen et al. 2004).

Next, we tested whether modulation of LBH expression in these cell lines had any effects on stemness by performing in vitro mammosphere assays. Remarkably, while siRNA-knockdown of LBH in MCF10A and 226L cells drastically reduced sphere formation (by ~80%; \( p<0.001 \)) (Fig. 4.11B), gain-of-function of LBH in HC11 cells significantly increased the propensity of these mammary epithelial cells to form spheres in non-adherent mammosphere cultures (>1.5 fold; \( p<0.05 \)) (Fig. 4.11E). We also noticed that sphere formation in human mammary epithelial cell lines correlated with the LBH expression levels, as 226L, which have 2.5 fold higher LBH mRNA levels as MCF10A, displayed a 2 fold increased sphere formation capacity relative to the latter cell line (Fig. 4.11A, B). Mirroring the striking effects of LBH on stemness, the stem-cell-specific isoform of p63, ΔNp63, was significantly downregulated in MCF10A and 226L cells upon LBH knockdown, whereas it was upregulated in HC11 cells ectopically expressing LBH (Fig. 4.11C, F). TAp63 was not expressed at detectable levels in any of these cell lines. Hence, the effects of LBH on this luminal p63 isoform could not be analyzed in these in vitro systems. However, mRNA expression of luminal differentiation marker ERα was significantly elevated in LBH-depleted MCF10A and 226L cells, whereas both ERα mRNA and protein levels were markedly downregulated in LBH-expressing HC11 as compared to vector-expressing cells (Fig. 4.11C,F; 4.12C,D). These data strongly suggest that LBH promotes stemness of normal mammary epithelial
cells, and does so at the mechanistic level by inducing the epithelial stem cell transcription factor ∆Np63 and repressing expression of hormone receptor ERα, which is essential for luminal cell proliferation and differentiation.

**Figure 4.11:** LBH increases sphere formation and ∆Np63 expression, while repressing ERα expression in mammary epithelial cell lines. (A) QPCR showing efficient depletion (>80%) of LBH mRNA expression in the human MCF10A and 226L mammary epithelial cell lines 3 days after transient transfection with LBH-specific siRNAs (siLBH) as compared to control scrambled siRNA (siCtrl)-transfected cells. (B) The same cells as in (A) were plated in non-adherent mammosphere cultures. siLBH transfected MCF10A and 226L cells showed a significant reduction in sphere formation as compared to siCtrl transfected cells after 7 days in suspension culture. ***p<0.001 siLBH vs. siCtrl transfected cells (triplicate samples; n=3 independent experiments). (C) QPCR analysis shows decreased ∆Np63 (~50%; p<0.05) and increased ERα (~30%; p=0.1; p<0.05) transcript levels respectively in both MCF10A and 226L siLBH groups as compared to siCtrl control cells. (D) QPCR quantification of Lbh transcript level in normal HC11 murine MECs stably transfected with empty pCDNA3 vector (HC11-Vector) or a pCDNA3-LBH expression plasmid (HC11-LBH) confirms exogenous overexpression of LBH in HC11-LBH cells. (E) Single cell suspensions of HC11-vector and HC11-LBH cells were grown for 7 days in mammosphere culture. HC11-LBH cells have a significantly increased sphere formation capacity (~50%; *p<0.05; n=3). (F) QPCR analysis shows significantly increased ∆Np63 transcript levels in HC11-LBH cells (~2.4 fold; p<0.05), while ERα levels are decreased (~50%; p<0.05) as compared to HC11-vector cells. All data represent mean ± SEM (n=3).
4.8 ∆Np63 acts downstream of LBH in promoting a basal stem cell phenotype and ERα repression

We next asked whether ∆Np63 is required for LBH-induced stemness of mammary epithelial cells. To this end, HC11-vector and HC11-LBH expressing cells were transiently transfected with siRNAs specific to p63 (Fig. 4.12). Knockdown of p63 led to a drastic reduction of ∆Np63 expression to virtually non-detectable mRNA and protein levels in both vector and LBH-expressing HC11 cells (Fig. 4.12A, D). To ascertain that depletion of p63 did not affect exogenous LBH expression in HC11 cells, qPCR analysis was performed. As shown in Fig. 8A, Lbh mRNA levels were not significantly altered (p=0.1) in HC11-vector and HC11-LBH cells transfected with p63-specific siRNA as compared to siCtrl transfected cells.

However, depletion of p63, significantly abolished the increase in sphere formation observed for exogenous LBH-expressing cells (Fig. 4.11E, 4.12B), suggesting that ∆Np63 is indeed required for the stem cell-promoting effects of LBH. Moreover, mRNA levels of basal K5, which were increased by exogenous LBH expression in HC11 (Fig. 4.12C, D), were significantly decreased upon p63 knockdown, reflective of K5 being a ∆Np63 target gene (Romano, Ortt et al. 2009). Conversely, p63 knockdown led to the de-repression of ERα in LBH-expressing HC11 cells (Fig. 4.12C, D), indicating that repression of ERα by LBH may be mediated through ∆Np63. Repression of ERα by LBH and ∆Np63
appears to be specific because expression levels of luminal marker K8 did not change significantly upon p63 knockdown (Fig. 4.12C, D).

Collectively, our data provide the first evidence of an essential mechanistic function of LBH in mammary stem cell regulation upstream of stem cell transcription factor ΔNp63, by promoting a basal MaSC phenotype. Conversely we found that LBH via ΔNp63 represses luminal differentiation and the expression of luminal-specific steroid hormone receptors (Fig. 4.13).

Figure 4.12: ΔNp63 is required for LBH-induced ‘stemness’ and repression of ERα expression in HC11 mammary epithelial cells. (A) QPCR analysis of relative ΔNp63 and Lbh transcript levels in HC11-vector and HC11-LBH mammary epithelial cells 3 days after transient transfection with scrambled control (siCtrl) or p63-specific (sip63) siRNAs. Values were normalized to Gapdh and represent mean ± SEM (n=3). (B) Three days post siRNA treatment cells were plated in triplicates in mammosphere suspension cultures. The significant (p<0.01) increase in sphere formation capacity observed for LBH-overexpressing HC11-LBH cells relative to HC11-vector control cells (see Fig. 7B) is abrogated by p63 knockdown (>50%; ***p<0.001; HC11-LBH+siCtrl vs. HC11-LBH+sip63), while HC11-vector cells displayed a modest but insignificant reduction in sphere formation upon p63 depletion. (C) QPCR analysis of ERα, K5, and K8 expression in HC11-vector and HC11-LBH cells revealed a significant derepression of ERα mRNA expression (p<0.05; HC11-LBH+siCTRL vs. HC11-LBH+sip63) and decrease in basal K5 expression (p<0.001; HC11-LBH+siCTRL vs. HC11-LBH+sip63) in HC11-LBH cells after p63 knockdown, luminal K8 levels did not significantly change. (D) Western blot analysis of HC11-vector and (V) and HC11-LBH (L) cell extracts 3 days post siRNA transfection shows efficient knockdown of ΔNp63 isoform (a, b, g) expression, de-repression of ERα and a reduction in basal K5 expression in sip63 transfected HC11-LBH cells. Actin was used as a loading control. Asterisks demarcate unspecific bands.
4.9 Conclusions

Since the function of LBH was unknown, and given the importance of LBH as a potential novel biomarker and therapeutic target for triple negative breast cancer (Rieger, Sims et al. 2010), it was important to determine the role of LBH in normal MG development. Therefore, we generated both ubiquitous Rosa26-Cre (Lindley and Briegel 2013) and epithelial-specific Keratin14-Cre;LbhloxP/loxP
knockout mice in order to investigate the effects of loss of LBH function during MG development. In this study we have identified the first essential role of the WNT/β-catenin-induced transcription cofactor LBH in the regulation of MaSC function and maintenance; as well as in epithelial lineage specification/differentiation. Our findings indicate that LBH may represent a new essential regulator of basal stem cells in the mammary gland through a novel mechanism whereby LBH acts upstream of ΔNp63.

We show that endogenous LBH was most highly expressed in the basal/MaSC compartment of the ducts and TEBs/alveoli of both the mouse and human mammary gland as evident by IHC analysis and abundant expression of Lbh mRNA specifically in the MaSC-enriched CD29hiCD24+ basal epithelial subpopulation of virgin mice (Fig. 4.1). This finding is consistent with previous RNA *in situ* hybridization analysis by our lab (Rieger, Sims et al. 2010), as well as recent gene expression profiling studies suggesting that LBH is one of few markers specifically and consistently expressed in both mouse and human purified MaSC populations (Lim, Wu et al. 2010). This, together with expression of LBH in numerous stem cell niches during embryonic development: in pluripotent blastomeres of Xenopus embryos at early cleavage stages (Paris, Osborne et al. 1988, Paris and Philippe 1990) and the dorsal Hensen’s node in chick (Alev, Wu et al. 2010), which acts as an organizer during gastrulation and gives rise to mesodermal progenitors (Nakaya and Sheng 2009), prompted us to investigate LBH as a potential functional regulator of tissue-specific stem cells using the MG as a model system.
MG development is a complex, highly orchestrated process directed by endocrine signals which regulate and act in concert with a host of transcriptional regulators to control proliferation and mediate cell lineage specification (Watson and Khaled 2008). A population of self-renewing MaSCs lies at the top of a hierarchy of cell lineages within the MG and it has been shown that only this small minority of cells found to reside in the basal epithelial layer are capable of recapitulating the mammary tree when transplanted in vivo (Deome, Faulkin et al. 1959, Shackleton, Vaillant et al. 2006, Stingl, Eirew et al. 2006, Van Keymeulen, Rocha et al. 2011). During post-natal development MaSCs in the basal layer of terminal end buds (TEBs), also referred to as ‘cap cells’ undergo massive proliferation resulting in ductal elongation into the mammary fat pad (Watson and Khaled 2008). Both ubiquitous (Rosa26-Cre) and epithelial-specific (Keratin14-Cre) directed loss of Lbh lead to a significant delay in pubertal MG outgrowth, providing preliminary evidence that loss of Lbh affects the functionality of MaSC that mediate outgrowth of the mammary epithelium into the fat-pad during puberty: (Fig. 4.3C,D; 4.4C,D). This epithelial growth retardation was accompanied by a greater than 25% reduction in the number of actively proliferating (Ki67+) cells in TEBs of K14-Cre;LbhloxP/loxP mutant MGs; which may also be indicative of MaSC dysfunction either by reduced number, functionality or both (Fig. 4.3E,F). Concurrent with this notion, MGs from Lbh null mice were found to have a drastic reduction in the MaSC enriched basal population (CD29hiCD24+) (Fig. 4.9A,B). However, this substantial decrease in the basal/MaSC enriched epithelial population in Lbh deficient MGs most likely was not
due to increased cell death, as these mice had normal low apoptosis rates. These findings rather led us to hypothesize that the reduction in MaSC might be due to non-regenerative differentiation and prompted us to consider the effect LBH may be having on the self-renewal and differentiation potential of MaSCs.

Histological analysis revealed aberrant luminal characteristics in the cellular morphology of the basal epithelial layer of Lbh deficient MGs providing preliminary evidence that lineage specification may be disrupted in these cells in the absence of LBH (Fig. 4.5A, 4.7A). IHC and qPCR analysis further revealed decreased protein and mRNA expression of basal Keratin 5 in the basal epithelial layer, while conversely a marked and significant increase in ERα+/PR+ cells in the luminal layer of Lbh deficient MGs was observed (Fig. 4.5B,C; 4.6; 4.7B,C). These findings support the idea that LBH may be regulating basal-luminal lineage specification by promoting a more basal undifferentiated cellular phenotype. In keeping with this notion, in vitro differentiation of Lbh-deficient CD29hiCD24lo MaSCs resulted in a majority of luminal epithelial cells at the expense of basal/myoepithelial cells indicating that loss of Lbh indeed results in MaSC differentiation dysfunction and a bias towards a more luminal phenotype (Fig. 4.9F,G; 4.10D,E).

An increased tendency of MaSCs to differentiate along the luminal lineage is also supported by the observed thickening of the luminal epithelium in Lbh deficient glands using H&E staining (Fig. 4.5A; 4.7A) and FACS analysis, which demonstrated a reciprocal increase in luminal (CD29loCD24hi) cells in response to the significant decrease in basal/ MaSC enriched (CD29hiCD24+) cells (Fig.
4.9A,B; 4.10A). One likely mechanism by which MaSC function and differentiation could be affected in the absence of \(Lbh\) is by modulation of other transcription factors involved in maintenance and cell specification. One such factor: \(\Delta Np63\), which is known for its role in maintaining MaSCs and basal epithelial phenotype, displays strikingly similar loss-of-function phenotypes to that of \(Lbh\) (Nylander, Vojtesek et al. 2002, Li, Li et al. 2006, Li, He et al. 2007, Senoo, Pinto et al. 2007, Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010). Indeed, \(\Delta Np63\) was found to be drastically decreased both at the protein and mRNA levels in \(Lbh\)-deficient MGs (Fig 4.5C; 4.8). The decrease in \(\Delta Np63\) levels in \(Lbh\)-deficient MaSC could be responsible for a range of observed phenotypes in our model, as \(\Delta Np63\) regulates cell proliferation, stem cell self-renewal, lineage specification, and maintenance of a basal phenotype in the MG (Nylander, Vojtesek et al. 2002, Li, Li et al. 2006, Li, He et al. 2007, Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010).

Moreover, knockdown of \(LBH\) in two human MEC lines (MCF10A and 226L) resulted in a significant reduction of \(\Delta Np63\), whereas overexpression of LBH in mouse MECs (HC11) resulted in an increase in \(\Delta Np63\) levels (Fig. 4.11). Of note, P63 encodes two different isoforms that are transcribed from alternate promoters: \(\Delta Np63\) and TAp63, which are expressed in the MaSC compartment and luminal cell compartment of the MG respectively (Yang, Kaghad et al. 1998, Nylander, Vojtesek et al. 2002, Li, Li et al. 2006, Li and Prives 2007, Yalcin-Ozuysal, Fiche et al. 2010). Given the previously established role of LBH as a transcriptional regulator \textit{in vitro} and \textit{in vivo} (Briegel and Joyner 2001, Briegel,
Baldwin et al. 2005, Conen, Nishimori et al. 2009) it is plausible that LBH may be acting at the promoter level to modulate ΔNp63 expression either through repression of the TA\(\text{p63}\) promoter, activation of the ΔNp63 promoter or a combination thereof. Our *in vivo* data would support this notion, as MECs from *Lbh* deficient mammary glands display decreased ΔNp63 and increased TA\(\text{p63}\) both at the mRNA and protein levels. The potential effects of LBH on p63 promoter switching may be either direct through complex formation with other transcription factors or indirect through regulation of signaling pathways, such as Hedgehog or Notch, which have been shown to suppress ΔNp63 and conversely, induce TA\(\text{p63}\) expression to promote luminal progenitor cell elaboration (Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010).

Concomitant with a decrease in ΔNp63 expression, knockdown of LBH in human MECs reduced mammosphere formation and increased ER\(\alpha\) mRNA expression (Fig. 4.11A-C). In contrast, overexpression of LBH in ER+ luminal MECs (HC11) lead to increased mammosphere formation and decreased ER\(\alpha\) mRNA expression (Fig. 4.11D-F). Given the observed LBH dependent ΔNp63 expression pattern, we posited that the effects of LBH on basal stem cell maintenance and repression of luminal differentiation might be mediated through ΔNp63. Remarkably, knockdown of ΔNp63 in LBH-overexpressing MECs (HC11) not only abrogated the LBH-mediated increase in mammosphere formation, but also lead to significant derepression of ER\(\alpha\) expression, restoring ER\(\alpha\) mRNA and protein levels to levels observed in control MECs (Fig. 4.12). Previous studies have demonstrated ΔNp63 is involved in regulating the expression of a
myriad of genes involved in cell proliferation, adhesion, and signal transduction (Wu, Wang et al. 2003, Carroll, Carroll et al. 2006). ΔNp63 has also been shown to directly induce expression of basal keratins, including Keratin 5 (Romano 2009). It, therefore, stands to reason that the aberrantly decreased expression of Keratin 5, as well the increased expression of ERα observed in both our in vivo and in vitro Lbh loss of function models may be due to ΔNp63. Loss of ΔNp63 expression, furthermore, may be responsible for the observed decrease in the stem cell activity of Lbh deficient MECs and their propensity to differentiate in a non-regenerative fashion predominantly into luminal cells; thus delineating a novel mechanism of MaSC control in the MG.

Intriguingly, studies by Badders et al., 2009 have shown that ΔNp63 expression in basal MECs is positively regulated by the WNT/b-catenin pathway. ΔNp63 is reduced in Lrp5+/− MECs, whereas it is, similar to Lbh, overexpressed in tumors from MMTV-Wnt1 transgenic mice (Badders, Goel et al. 2009, Rieger, Sims et al. 2010). However, it is not known, whether ΔNp63 is a direct β-catenin/TCF target gene. Since LBH is a direct Wnt target gene (Rieger, Sims et al. 2010), our finding that LBH induces ΔNp63 expression and that the stem cell promoting effects of LBH are dependent on ΔNp63 suggests the following novel molecular pathway: WNT-LBH-ΔNp63-stemness (Fig. 4.13). Futures studies will need to determine whether LBH is an effector of WNT in MaSC development and oncogenesis, such as the use of genetic crosses of Lbh null mice with MMTV-Wnt1 transgenic mice (Tsukamoto, Grosschedl et al. 1988). Of note, knockout of the histonemethylase Pygo2, another WNT target gene implicated in cell-intrinsic
MaSC control (Gu, Sun et al. 2009), failed to prevent the accumulation of stem/progenitor-like cells that is typical for MMTV-Wnt1 transgenic MGs and does not affect canonical Wnt signaling output when crossed into MMTV-Wnt1 mice (Watanabe, Fallahi et al. 2013). Thus, it would be highly interesting to evaluate whether LBH may act as a cell-intrinsic factor mediating the stem cell effects of Wnt. Molecular studies to identify transcriptional targets and interacting partners of LBH are also of great importance as this information will shed light on the mechanism by which LBH may regulate p63 and MaSC function.
Role of LBH in Mammary Oncogenesis

Chapter 5: LBH is a Marker for Triple-Negative Breast Cancer and is Essential for Promoting a Malignant Breast Cancer Stem Cell Phenotype*

*Portions of this chapter were previously published (Lindley 2010)
5.1 Background

Our lab has previously shown that aberrant LBH overexpression in breast cancer correlates with WNT pathway hyperactivation specifically in aggressive basal subtype, triple negative breast cancers (TNBC) (Rieger, Sims et al. 2010), which are enriched in breast cancer stem cells (BCSCs) (Honeth, Bendahl et al. 2008). Understanding the molecular mechanisms underlying the formation and maintenance of BCSCs is therapeutically advantageous given that breast cancers enriched in these cells (basal subtype) have the poorest prognosis and no targeted therapies (Honeth, Bendahl et al. 2008). The exact mechanisms resulting in the genesis of BCSCs in vivo is still uncertain; however it has been shown in recent years that EMT during tumor progression can induce the dedifferentiation of differentiated breast epithelial cells into cells with a stem cell phenotype (Frixen, Behrens et al. 1991, Yang, Mani et al. 2004, Mani, Guo et al. 2008, Morel, Lievre et al. 2008, Sabbah, Emami et al. 2008, Taube, Herschkowitz et al. 2010). Epithelial-mesenchymal transition (EMT) is a morphogenetic program crucial for epithelial cell plasticity during embryogenesis, wound healing and tissue homeostasis (Thiery, Acloque et al. 2009). EMT is characterized by the loss of epithelial cell-to-cell contacts with a decrease in epithelial adhesion molecule E-cadherin, loss of epithelial cell polarity, a major reorganization of the cytoskeleton, an increase in mesenchymal marker expression, and gain of a fibroblastic, motile cell phenotype (Thiery, Acloque et al. 2009).
We have recently found that LBH is rapidly induced during TGFβ mediated EMT induction (unpublished) and dedifferentiation of primary human mammary epithelial cells (Lindley and Briegel 2010) a finding that implicates LBH in the genesis of BCSCs. Furthermore, preliminary results from the thesis work of former graduate student Megan Rieger have suggested that LBH is required for BCSC maintenance (Rieger 2010). Additional evidence for a role of LBH in the genesis and maintenance of BCSCs is provided herein and highlights a novel mechanism for LBH in inducing expression of key mammary stem cell (MaSC) maintenance factor ΔNp63 and repressing the good-prognosis marker Estrogen Receptor alpha (ERα) similar to its role in normal MaSC biology (Chapter 4).

5.2 LBH is Upregulated During EMT-Associated Dedifferentiation of HMEC

Aberrant reactivation of latent developmental signaling pathways and TFs in tumor cells has been associated with, and shown to play causal roles in advanced-stage, invasive cancers (Briegel 2006, Ben-Porath and Tellegen 2008, Yang and Weinberg 2008). TGFβ is a potent EMT-promoting cytokine in various physiological and pathological settings that is expressed at chronically high levels in invasive tumors, and in inflamed or fibrotic tissues (Zavadil and Bottinger 2005, Thiery, Acloque et al. 2009). TGFβ can promote neoplastic progression and metastasis of transformed epithelial cells (Cui, Fowlis et al. 1996, Oft, Heider et al. 1998), due to its ability to induce EMT (Oft, Peli et al. 1996). Overexpression of embryonic transcription factors (TFs), which can act downstream of TGFβ, such as ZEB1, SNAIL, TWIST, GOOSECOID (GSC), and FOXC2, have been
shown to be sufficient to induce EMT in immortalized or transformed MEC (Yang, Mani et al. 2004, Eger, Aigner et al. 2005, Hartwell, Muir et al. 2006, Mani, Yang et al. 2007, Mani, Guo et al. 2008). Given the synergistic nature of many developmental pathways, our findings that Lbh plays a role in maintaining stem/progenitor cell self-renewal (Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009, Rieger, Sims et al. 2010) and that LBH is preferentially overexpressed in poorly differentiated tumors harboring large populations of BCSCs we asked whether LBH might also be downstream of TGFβ mediated EMT and subsequent dedifferentiation.

Using an in vitro system, we performed a detailed time course analyses of the cellular and molecular events with focus on the induction kinetics of EMT-promoting TFs during TGFβ mediated EMT in primary human mammary epithelial cells (HMEC) (Lindley and Briegel 2010). Importantly, we demonstrated that TGFβ-mediated EMT of HMEC occurs in three distinct phases that are governed by a TF hierarchy. Loss of epithelial cell polarity (ZO-1), and acquisition of mesenchymal marker (Vimentin, Fibronectin) expression are immediate-early events, whereas switching from E-cadherin to N-cadherin protein expression occurs only after EMT-like morphological changes become apparent. The kinetics of TF induction suggests that ZEB1 and SNAIL mediate early EMT induction reinforced by ZEB2, while GOOSECOID and FOXC2 may play a role in EMT maintenance. TWIST and SLUG were not significantly induced in this system. Furthermore, we showed for the first time that normal HMEC acquire a CD44+CD24−/low stem cell phenotype during a third phase of EMT that is
characterized by maximum TF expression levels. Using this system we also found that LBH is rapidly induced during TGFβ-mediated EMT in primary HMECs similar to ZEB1 and this rapid induction is sustained throughout the treatment time course.

5.2.1 TGFβ efficiently induces EMT-like morphological changes in HMEC

To investigate whether normal HMEC are capable of undergoing an EMT program in response to TGFβ, we cultured early passage primary finite lifespan HMEC in the absence or presence of 5 ng/ml TGFβ1. Cells were monitored for phenotypic changes over a period of 12 days. In contrast to control cells, TGFβ-treated HMEC exhibited marked growth inhibition within 48 h, in keeping with previous studies showing that TGFβ promotes growth inhibition of these cells (Hosobuchi and Stampfer 1989, Sandhu, Garbe et al. 1997). Normal HMEC have a characteristic cobblestone-like appearance and tend to form clusters when grown in culture due to intercellular junctions that are responsible for maintaining epithelial cellular polarity and adhesion (Fig. 5.1A). However, this cell morphology began to change into a more fibroblastic phenotype at approximately 2–3 days after TGFβ treatment and cells became noticeably more dispersed (Fig. 5.1A; data not shown). Furthermore, after 6 days of TGFβ treatment HMEC grew in size and many cells formed a “leading edge” characteristic of motile cells. By 9–12 days, TGFβ-treated HMEC were a heterogeneous population of mostly fibroblastic cells (Fig. 5.1A). To assess whether the observed morphological changes were the result of an EMT, we next analyzed expression and
localization of epithelial/mesenchymal markers using IF analysis. As shown in Fig. 5.1B, after treatment with TGFβ, epithelial markers E-cadherin and ZO-1 were both mislocalized and their expression was reduced, such that these proteins were completely absent from the cell membrane. Conversely, all of the mesenchymal markers examined, N-cadherin, Vimentin, and Fibronectin, were visibly increased and properly localized after TGFβ treatment (Fig. 5.1B). Thus, the phenotypic changes occurring upon treatment with TGFβ indicate that TGFβ alone is competent to induce EMT in normal finite lifespan HMEC.

**Figure 5.1:** TGFβ induces EMT-like morphological changes in finite lifespan HMEC. (A) Brightfield microscopy images (20× magnification) of HMEC without (control) or with 5 ng/ml TGFβ1 (+TGFβ) for the indicated time points. Phenotypic changes become apparent at 3 days (d) of TGFβ treatment. (B) Immunofluorescence analysis (63× magnification) of EMT marker expression and localization in control cells and cells treated with TGFβ for 9–12 days. Note the repression of epithelial markers, induction of mesenchymal markers, and the major reorganization of the actin cytoskeleton (phalloidin staining). (Lindley and Briegel 2010)
5.2.2 Loss of epithelial cell polarity and mesenchymal marker induction are immediate-early events in TGFβ-induced EMT of HMEC

To further assess the kinetics of EMT induction in TGFβ-treated HMEC, we performed a detailed time course analysis of EMT marker expression using qPCR and Western blot analysis. Repression of E-cadherin was observed as early as 6 h at the mRNA level after TGFβ induction reaching maximal repression (6.3-fold) at 9 days. However, E-cadherin protein levels were not reduced before 3 days, when morphological changes occurred (Fig. 5.1 and Fig. 5.2B). In contrast, the epithelial cell polarity marker ZO-1 was repressed at both the mRNA and protein levels as early as 6 and 24 h, respectively, preceding phenotypic changes with levels declining further (6.5-fold) during the 12 day period of our analysis (Fig. 5.2A and B). Moreover, a rapid increase in expression of mesenchymal markers Vimentin and Fibronectin was observed as early as 6 h reaching maximal levels at 3 days of TGFβ treatment. Early induction of Fibronectin is not surprising, as TGFβ has previously been shown to induce extracellular matrix proteins in this cell system (Stampfer, Garbe et al. 2003). In addition, expression of N-cadherin was rapidly induced at the mRNA level as early as 6 h followed by a linear increase to over 600 fold until 12 days (Fig. 5.2A). However, N-cadherin protein expression became detectable only after 3 days of TGFβ treatment. These observations are in accordance with previous studies suggesting that switching from epithelial E-cadherin to mesenchymal N-cadherin expression is not essential for the morphological changes during TGFβ-
induced EMT (Maeda, Johnson et al. 2005). In contrast, the immediate downregulation of ZO-1 in conjunction with upregulation of intermediate filament Vimentin upon TGFβ induction indicates that deterioration of epithelial cell polarity and cytoskeletal reorganization may play significant roles in EMT cell morphology changes of

![Image of graph and Western blot](image)

**Figure 5.2:** Loss of epithelial cell polarity and mesenchymal marker induction are immediate-early events in TGFβ-induced EMT of HMEC. (A) qPCR analysis of EMT marker expression in TGFβ-treated HMEC. Values were normalized to GAPDH mRNA levels and represent fold change as compared to control untreated HMEC at the indicated time points. The data are represented as mean ± SEM. (B) Western blot analysis of whole cell lysates of untreated control (ctrl) and TGFβ-treated (+TGFβ) HMEC at the indicated time points. Changes in ZO-1, Fibronectin, and Vimentin protein expression are detectable within 6–24 h of TGFβ treatment. In contrast, E-cadherin and N-cadherin levels do not change until after 3 days when morphological changes (Fig. 5.1A) occur. (Lindley and Briegel 2010)
normal HMEC. Most importantly, the rapid kinetics of initiation of an EMT program in response to TGFβ indicates that HMEC possess the innate ability to undergo EMT. Thus, arguing against the possibility that the observed “EMT-phenotype” may be an artifact due to the growth-inhibitory effects of TGFβ on normal HMEC (Hosobuchi and Stampfer 1989, Sandhu, Garbe et al. 1997), which could lead to overgrowth of a potentially pre-existing mesenchymal subpopulation in primary HMEC cultures (Hosobuchi and Stampfer 1989).

5.2.3 HMEC acquire a CD44^+CD24^-/low stem cell phenotype during TGFβ-mediated EMT

To further assess whether EMT in normal HMEC correlates with a gain of “stem-ness”, as previously reported for HMLE and HMLER (Mani, Guo et al. 2008, Morel, Lievre et al. 2008), we next examined expression of cell surface markers CD44 and CD24 in TGFβ-treated HMEC using FACS analysis (Fig. 5.3). Parental untreated HMEC contained two subpopulations: a major subpopulation (93%) of CD44^{low}/CD24^{+/high} and a minor subpopulation (4.4%) of CD44^{high}/CD24^{+} cells (Fig. 5.3A and B). However, in HMEC treated with TGFβ there was a dramatic shift from a CD44^{low} to a CD44^{high} phenotype. This change was first noticeable between 2–3 days, coinciding with morphological changes, and became gradually more pronounced until 12 days when the CD44^{high} population reached a maximum of 53%, whereas the CD44^{low} population declined to 32% (Fig. 5.3A and B). In addition, a third population of CD44^{+}/CD24^{-/low} cells (10%) became apparent at this time point increasing
further to 21% by 30 days of TGFβ treatment (Fig. 5.3 and Fig. 5.4; data not shown). As the CD44+/CD24−/low phenotype has been associated with normal mammary epithelial stem cells (Sleeman, Kendrick et al. 2007), as well as with tumor-initiating, metastatic breast cancer stem cells (Al-Hajj, Wicha et al. 2003, Fillmore and Kuperwasser 2008), we conclude that normal HMEC, like their

**Figure 5.3**: HMEC acquire a CD44+/CD24−/low stem cell phenotype during TGFβ-mediated EMT. (A) FACS analysis of cell surface markers CD44 and CD24. Untreated control HMEC consist of a major CD44low/CD24+ (I) and a minor CD44high/CD24+ (II) cell population. TGFβ treatment significantly shifts cells from a CD44low to a CD44high expression profile after day 3. At 12 days a third population of CD44+/CD24−/low cells (III) appears, a phenotype in keeping with normal and neoplastic mammary epithelial stem cells. (B) Table showing quantification of the results depicted in A. (Lindley and Briegel 2010)
transformed counterparts dedifferentiate during EMT into cells with increased stem-like properties. Moreover, our data demonstrate for the first time that CD44+/CD24−/low stem-like cells arise during the late phases of EMT.

5.2.4 Kinetics of EMT-promoting TF expression demarcates three different phases of EMT in TGFβ-treated HMEC

To begin to investigate the transcriptional programs that may control EMT in normal HMEC downstream of TGFβ, we evaluated the kinetics of induction of key EMT-activating TFs SNAIL, SLUG, ZEB1, ZEB2 (SIP1), TWIST, GSC, and FOXC2 (Thiery, Acloque et al. 2009) in TGFβ-treated HMEC using qPCR analysis (Fig. 5.4A). As shown in Fig. 5.4A, ZEB1 was the first TF to be markedly induced (6.5-fold) followed by SNAIL (2.1-fold) within 6 h of TGFβ treatment; mRNA levels of ZEB1 and SNAIL increased further until 3 days (15 and 10.2-fold, respectively), when phenotypic changes were observed. The rapid induction kinetics of these TFs is in line with the fact that ZEB1 and SNAIL are TGFβ target genes (Cho, Baek et al. 2007, Shirakihara, Saitoh et al. 2007). Thereafter, ZEB1 and SNAIL expression levels showed a modest decline (10.4 and 6.2-fold induction, respectively) until they culminated in a maximal increase (32- and 27-fold, respectively) at 12 days after TGFβ induction (Fig. 5.3A). The onset of ZEB2 induction (2.1-fold) occurred at 24 h of TGFβ treatment, possibly as a consequence of stabilization of its mRNA by SNAIL (Beltran, Puig et al. 2008), reaching highest induction levels (17-fold) at 12 days (Fig. 5.4A and B). In contrast, GSC and FOXC2 were not induced (3.7- and 2.3-fold, respectively).
Figure 5.4: Kinetics of EMT-promoting TF induction demarcates three different phases of EMT in TGFβ-treated HMEC. (A) qPCR analysis of untreated control (ctrl) and TGFβ-treated HMEC over a period of 12 days (d). Values represent fold change as compared to control cells. GAPDH mRNA was used as a normalization control; data are represented as mean ± SEM. ZEB1 and SNAIL are the first and most highly induced TFs. ZEB2 induction commences at 24 h. In contrast, GSC and FOXC2 are not markedly induced before 6 days, which is after EMT-like morphological changes occur (Fig. 5.1A). TWIST and SLUG are not significantly induced. (B) Model depicting the three different phases (induction, maintenance, and dedifferentiation) of EMT in HMEC. (Lindley and Briegel 2010)
before 6 days of TGFβ treatment and mRNA levels of these TFs only slightly increased (6.3- and 2.6-fold, respectively) through 12 days (Fig. 5.4A and B). Moreover, SLUG was only modestly induced (~2-fold) throughout the course of the experiment starting at 48 h after TGFβ induction, whereas TWIST was not induced in this system (Fig. 5.4A).

These data suggest that TGFβ-mediated EMT in HMEC is regulated by a hierarchy of EMT-promoting TFs led by ZEB1 and that EMT occurs in at least three distinctive phases that appear to be controlled by different combinations and concentrations of these TFs (Fig. 5.4B). Based on their rapid kinetics and magnitude of induction, ZEB1 and SNAIL appear to be key activators of the EMT program in primary HMEC. Both ZEB1 and SNAIL are direct E-cadherin repressors and also repress cell polarity genes, as well as induce transcription of Vimentin (Batlle, Sancho et al. 2000, Eger, Aigner et al. 2005, Whiteman, Liu et al. 2008), suggesting that these TFs mediate the observed immediate-early changes in EMT marker expression during the first phase of EMT induction (Fig. 5.2 and Fig. 5.4B). Subsequent upregulation of ZEB2, another direct E-cadherin repressor (Comijn, Berx et al. 2001), just prior to when EMT-like morphological changes occur, may indicate that ZEB2 cooperates with ZEB1 and SNAIL during EMT induction facilitating phenotypic changes and cadherin switching (Comijn, Berx et al. 2001), which demarcate the beginning of the second phase of EMT (Fig. 5.4B). In contrast, GSC, and to a lesser extent FOXC2, both of which repress E-cadherin transcription only indirectly (Mani, Guo et al. 2008), may play a role in EMT maintenance, as their induction occurred after EMT phenotypic
changes. Finally, acquisition of a stem-like CD44+/CD24−/low molecular signature of HMEC coincided with maximal levels of all of these EMT-activating TFs during the third phase of EMT (Fig. 5.4B). Although ZEB1 and SNAIL are known to confer stem-like properties on preneoplastic and neoplastic epithelial cells (Mani, Guo et al. 2008, Wellner, Schubert et al. 2009), our results suggest that their cooperation with other EMT-activating TFs may be required for HMEC dedifferentiation.

5.2.5 LBH is Rapidly Induced During TGFβ-mediated EMT in HMECs

We wanted to explore whether LBH was also induced during EMT mediated dedifferentiation using the model system described here. Interestingly, we found LBH mRNA to be rapidly induced (>5 fold) within 6 hours of TGFβ treatment (Fig 5.5A) resembling the induction kinetics observed for ZEB1 and SNAIL1 (Fig. 5.4A,B). The rapid kinetics of LBH induction also suggested that LBH may be a direct transcriptional target of the TGFβ pathway. LBH reached its maximal induction after only 24 hours of TGFβ treatment (~30 fold) and unlike any of the other TFs queried, this elevated level of mRNA expression was sustained throughout the 12-day time course of the experiment (Fig. 5.4A, 5.5A). The induction of LBH protein expression following TGFβ treatment displayed a similar kinetics to the rapid LBH mRNA induction (Fig. 5.5A,B) and immunofluorescence revealed that LBH protein was primarily localized to the nucleus, suggestive of a transcriptionally active role for LBH during this process (Fig 5.5C). *In silico* analysis revealed a putative conserved SMAD binding
Figure 5.5: LBH is induced during EMT-associated dedifferentiation of HMECs and contains a conserved, functional SBE in the upstream promoter region. (A) qPCR of FOXC2, ZEB1 and LBH expression in TGFβ treated (5ng/ml) HMECs over a 12 day time course showing rapid and sustained induction of LBH mRNA to even greater levels than ZEB1 while FOXC2 is only moderately induced after 6 days. (B) Western Blot analysis of E-cadherin (E-Cad), Vimentin and LBH expression in TGFβ treated (5ng/ml) HMECs over a 12 day time course showing rapid and sustained induction of LBH and Vimentin protein prior to the reduction in E-cad protein. (C) Immunofluorescence of HMEC treated with TGFβ for 12 days showing nuclear localization of LBH protein (LBH affinity purified rabbit polyclonal antibody). (D) Schematic of the Lbh promoter region outlining the putative conserved SMAD-binding element (SBE) upstream of the transcriptional start site (-8998 human, -8986 mouse) as well as luciferase constructs with (ΔSX) or without (ΔBX) the putative SBE. (E) HC11 cells were used to measure luciferase fold trans-activation of empty vector (pGL3), ΔSX and ΔBX (described in D) upon co-transfection with pCMV-flag-SMAD3 and 3 hour treatment with exogenous TGFβ (5ng/ml).
element in both human and mouse (-8998; 8986 bp respectively) upstream of the \textit{Lbh} transcriptional start site (Fig. 5.6D). To test the functionality of the putative SBE, transient reporter assays were performed using HC11 MECs transfected with luciferase promoter constructs containing (ΔSX) or lacking (ΔBX) the putative SBE (Fig. 5.6 D) as well as pGL3 (empty vector) in combination with a pCMV-flag-SMAD3 construct and 3hr treatment with TGFβ1 (5ng/ml; Fig. 5.6 D,E). Preliminary results demonstrated that the TBE containing ΔSX construct showed a 2.5 fold transactivation when co-transfected with pCMV-flag-SMAD3 and this activation was further enhanced upon treatment with TGFβ1 (Fig. 5.6E). In contrast, the pGL3 and ΔBX constructs were not significantly stimulated (Fig 5.6E) indicating that the putative SBE is indeed functional. Together this data indicates that LBH is a direct target not only of the WNT stem cell pathway but also the cooperating TGFβ pathway. Moreover, it is highly feasible that LBH is involved in both TGFβ-mediated EMT induction as well as in the dedifferentiation process that occurs with it.

5.3 \textit{LBH} is expressed in basal subtype, triple negative breast cancer (TNBC)

Meta-analysis previously identified \textit{LBH} as being overexpressed specifically in basal-subtype TNBCs and \textit{in situ} RNA hybridization analysis demonstrated \textit{Lbh} was overexpressed in Wnt-driven mammary tumors of \textit{MMTV-Wnt1} mice (Tsukamoto, Grosschedl et al. 1988, Li, Welm et al. 2003, Liu, McDermott et al. 2004, Honeth, Bendahl et al. 2008, Rieger, Sims et al. 2010, Watanabe, Fallahi
et al. 2013). This finding, however, had not yet been verified at the protein level in tissue samples. To confirm expression of LBH specifically in basal subtype cancers at the protein level, a rabbit polyclonal anti-LBH antibody generated by our laboratory was affinity purified using recombinant LBH protein (see methods 2.2.8 for a detailed protocol) and carefully optimized for the use in IHC analysis of human and mouse paraffin embedded tissues. Preliminary IHC analysis of different human tumor subtypes confirmed that LBH protein is specifically overexpressed in tumor cells of basal subtype TNBC tumors (Fig. 5.6) as gene expression profiling would suggest (Rieger, Sims et al. 2010). HER2+ tumor cells were LBH-negative but contained LBH+ tumor associated fibroblasts (Fig. 5.6), while staining was virtually absent from most luminal ER+/PR+ tumors (Fig. 5.6).

![Figure 5.6](image)

**Figure 5.6:** LBH is highly expressed in tumor cells of poorly differentiated triple negative tumors. Immunohistochemistry of human breast tissue and tumors using affinity purified polyclonal rabbit LBH antibody. From the left to right: luminal subtype, hormone receptor positive human breast tumor which is LBH-, a HER2+ hormone receptor negative tumor which is only LBH+ in tumor associated fibroblasts (brown) and hormone receptor ‘triple negative’ breast cancer which is highly LBH+ (brown) in both the cytoplasm and nucleus of tumor cells and associated stroma.
5.4 LBH promotes stemness of human breast tumor cells

Previous WB and FACS analyses have shown that LBH expression correlates with a BCSC phenotype \((\text{CD44}^\text{hi}\text{CD24}^\text{lo})\) in TNBC cell lines and RNAi knockdown (KD) of LBH expression in TNBC cell lines results in a shift towards a more differentiated \((\text{CD44}^\text{hi}\text{CD24}^\text{hi})\) phenotype (Rieger 2010). KD of LBH also resulted in decreased colony formation in soft agar, indicating that depletion of LBH also reduced the tumorigenicity of cells. However, a careful functional analysis of the effects of LBH on BCSC activity has not yet been carried out therefore, mammosphere assays after RNAi mediated KD of LBH (Fig. 5.7A) were performed using three basal TNBC cell lines in which endogenous LBH is overexpressed (MDA-MB-231, HCC1187, and MDA-MB-157). Conversely, to assess if LBH can promote BCSCs formation, we overexpressed LBH (Fig. 5.7B) in both a luminal ER+ breast cancer cell line (MCF7; LBH-) and a basal, TNBC cell line (BT549; ER-; LBH-); which in comparison to most TNBC lines only have a small population of BCSCs.

![Figure 5.7: Lhb knockdown (KD) and overexpression efficiency. (A) Three TNBC cell lines that overexpress LBH (Rieger 2010) were transfected with scrambled control (siCtrl) or LBH specific (siLBH) siRNA in 2D culture. KD efficiency of LBH was assessed by qPCR analysis three day post siRNA transfection. (B) Overexpression levels of LBH in stably transfected MCF7 and BT549 cell lines (previously established by Megan Rieger). Normalization to GAPDH, values represent fold change as compare to siCtrl or vector controls (n=3).](image-url)
KD of LBH drastically reduced the number of mammospheres by 50% in all of the three tumor lines assessed (Fig. 5.8A), indicating that LBH plays a functional role in BCSC maintenance. Conversely, overexpression of LBH in both MCF7 and BT549 resulted in a significant increase in mammosphere formation by >100% and 50% respectively (Fig. 5.8B), suggesting that in addition to a maintenance role LBH may play a role in promoting BCSCs formation.

**Figure 5.8:** LBH promotes stem cell activity in breast cancer cell lines. (A) Three TNBC cell lines that overexpress LBH (Rieger 2010) were transfected with scrambled control (siCtrl) or LBH specific (siLBH) siRNA in 2D culture. 3 days after siRNA transfection cells were dissociated into single cell suspensions and plated in non-adherent mammosphere culture. LBH-depleted TNBC cell lines showed a significant (**p<0.01) reduction in their sphere forming capacity as compared to siCtrl-transfected cells. (B) LBH was ectopically expressed in the basal ER- breast carcinoma cell lines BT549 and the luminal ER+ MCF7 cell line (Rieger 2010). Cells were dissociated to single cell suspensions and plated in non-adherent mammosphere culture. MCF7-LBH and BT549-LBH tumor cells showed a significant (**p<0.01; *p<0.05 respectively) increase in sphere forming capacity as compared to vector control cells.
5.5 LBH promotes a basal phenotype and represses luminal differentiation in breast cancer cell lines

LBH has been shown to have roles in modulating transcriptional activity in numerous contexts and model systems (Briegel and Joyner 2001, Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009, Rieger, Sims et al. 2010). Given our observation of a striking positive relationship between LBH and induction of ΔNp63 (Fig. 4.11-4.12) with subsequent repression of ERα in normal MaSC at the transcriptional level, we sought to determine if the same relationship existed in breast cancer cells. To this end, RNA was collected from three TNBC cell lines (which overexpress endogenous LBH: MDA-MD-231; HCC1187; MDA-MB-157) (Rieger, Sims et al. 2010) three days post transfection of cells with scrambled control (siCtrl) or LBH specific (siLBH) siRNAs. Additionally, RNA was collected from MCF7-LBH and BT549-LBH expressing tumor lines and appropriate vector controls. cDNA was generated and qPCR analysis of ΔNp63 and ERα expression was performed.

Strikingly, four out of the five cell lines analyzed showed an LBH dependent regulation in transcript levels of both ΔNp63 and ERα (Fig. 5.9) with the only exception being MDA-MB-157 which despite efficient KD of LBH (Fig. 5.7A) displayed no changes in ΔNp63 or ERα mRNA. Upon KD of LBH in MDA-MB-231 and HCC1187 there was a significant decrease in ΔNp63 transcript levels (80% and 45% decrease respectively; Fig. 5.9A). In MCF-LBH and BT549-LBH however, a significant increase in LBH dependent ΔNp63 was observed (>4;
3.5 fold respectively; Fig. 5.9B) overall recapitulating the same positive relationship observed for LBH and p63 in primary MaSC and normal non-transformed human MEC. Furthermore, LBH KD resulted in increased expression of key luminal differentiation and prognostic marker ERα in both MDA-MD-231 and HCC1187 cells (Fig. 5.9C) while it lead to repression of ERα in MCF7-LBH (Fig. 5.9D). Of note, ERα expression was not or barely detectable in BT549 and MDA-MB-231 TNBC cell lines, which might be due to epigenetic suppression of ERα by hypermethylation of the ER promoter, which is commonly observed in basal B type tumors.

Figure 5.9: LBH induces ΔNp63 and represses ERα expression. (A) qPCR analysis showing decreased ΔNp63 expression in MDA-MB-231 and HCC1187 cells 3 days post KD of LBH. MDA-MB-157 displays no change despite efficient LBH KD (Fig. 5.6A). (B) qPCR analysis showing increased ΔNp63 expression in both stable LBH overexpressing MC7-LBH and BT549-LBH cell lines. (C) qPCR analysis showing increased ERα expression in both stable LBH overexpressing MC7-LBH and BT549-LBH cell lines. (D) qPCR analysis showing a downregulation of expression in the presence of stably overexpressed LBH in MCF7-LBH. ERα did not amplify in BT549 cells. Normalization to GAPDH, values represent what fold change in comparison to siCtrl, error bars represent SEM (n=3). **All p63 qPCR analysis was performed by Dr. Kevin Curtis (samples were prepared by Linsey E. Lindley).
5.6 LBH promotes breast cancer stem cell motility and invasiveness

Because BCSCs have been associated with increased motility and metastasis (Mani, Guo et al. 2008), we next assessed the effects of LBH modulation on the migration and invasion potential of breast cancer cell lines. Migration (*in vitro* transwell migration assays) and invasion assay (transwell matrigel assay) was performed by Dr. Bin Wang in our group. Both migration and invasion rates were significantly decreased in MDA-MB-231 and MDA-MB-157 upon KD of *LBH* (Fig. 5.10A,B) indicating that LBH had an effect on cell motility and the ability to invade surrounding areas in 3 dimensions. Conversely, MCF7-LBH and BT549-LBH tumor cells were significantly more migratory than respective vector control cells (Fig. 5.10A,B). In contrast, increased invasiveness was only observed for BT549-LBH tumor cells (Fig. 5.10B) likely due to the fact that MCF7 are inherently low-invasive and LBH overexpression alone was not sufficient to reverse this.

**Figure 5.10:** LBH increases motility and invasiveness of breast cancer cell lines. (A) Migration analysis showing KD of *LBH* significantly decreased migration through 8.0µm polycarbonate transwell inserts after 12 hours incubation for both MDA-MB-231 and MDA-MB-157 cells (**p<0.01; **p<0.01 respectively). Conversely overexpression of LBH results in significantly increased migratory capability in both MCF-LBH and BT549-LBH as compared to vector controls (*p<0.05; **p<0.01 respectively). Cells on inserts were stained with crystal violet and mounted prior to pictures being taken at 40x magnification. (B) 3D-Transwell invasion analysis showing KD of *LBH* results in significantly decreased invasiveness of MDA-MB-231 and MDA-MB-157 cells through matrigel coated 8.0µm polycarbonate inserts after 48hrs (*p<0.05; **p<0.01 respectively). Overexpression of LBH significantly increased in the invasive properties of BT549 cells into matrigel coated inserts (**p<0.01), while MCF7 were completely non-invasive regardless of LBH expression levels. Cells on inserts were stained with 1% toluidine blue/ 1% borax and mounted prior to pictures being taken at 40x magnification. **All migration and invasion assays were performed by Dr. Bin Wang.
5.7 Conclusions

We established a model for EMT induction in primary HMEC, which will provide a useful in vitro system to dissect the currently incompletely understood molecular mechanisms underlying EMT in normal breast epithelial cells. Taken together our data demonstrate that HMEC possess the innate ability to undergo a full EMT in response to TGFβ leading to acquisition of a stem cell-like phenotype, and that this program appears to be controlled by a defined hierarchy of EMT-activating TFs including LBH. As such, our study may have fundamental significance for understanding potential changes that might occur in normal breast epithelium under chronically elevated TGFβ levels, such as in a tumor microenvironment and especially in the genesis of cancer stem cells. Furthermore, we have demonstrated that LBH displays a rapid and sustained induction in this system (mRNA and protein; Fig. 5.5A,B) and LBH protein is localized to the nucleus of cells (Fig. 5.5C) indicating it may be contributing to the transcriptional signature mediating the EMT program.

In silico analysis using Scansite indicates that there is a conserved putative SBE in the promoter at -8998 bp in human, and -8986 in mouse upstream of the LBH transcriptional start site (Fig. 5.5D). Preliminary transient reporter assays indicate that this SBE is responsive to TGFβ stimulation implicating LBH as a direct TGFβ target and also explaining the observed rapid induction kinetics in this model (as early as 6 hours post TGFβ treatment; Fig. 5.5A,D,E). Immediate induction, similar to ZEB1 and SNAIL1 also indicates that LBH could be affecting early events in EMT especially in light of our recent
findings in which LBH has been demonstrated to induce ΔNp63, a stem cell transcription factor whose numerous variants have been implicated in EMT of various tissues and which has also been found to directly regulate members of the TGFβ family and superfamily (Section 4.7) (Lindsay, McDade et al. 2011, Balboni, Hutchinson et al. 2013). ΔNp63 has also been shown to regulate transcription of cell adhesion genes as well as genes involved in mesenchymal phenotype (Wu, Wang et al. 2003, Carroll, Carroll et al. 2006, Romano, Ortt et al. 2009) and furthermore is crucial to the maintenance of an undifferentiated stem cell phenotype especially in the mammary gland (Yalcin-Ozuysal, Fiche et al. 2010). Future studies will be needed to examine if ΔNp63 is induced in our HMEC EMT system and if LBH plays a functional role upstream of ΔNp63 in TGFβ-mediated EMT. This work expands on the already well defined regulation of LBH by the Wnt pathway (Rieger, Sims et al. 2010) and provides important new insight into the possible regulation of LBH by TGFβ a finding that is especially exciting given the recently demonstrated synergistic requirement of Wnt and TGFβ in maintenance of MaSC (Scheel, Eaton et al. 2011). Furthermore, hyperactivation of both of these signaling pathways may serve as potential mechanisms for deregulation of LBH in breast cancer and in the genesis and maintenance of BCSCs.

Previous studies performed by our laboratory found LBH to be overexpressed in both human breast tumors and Wnt-driven mammary tumors (MMTV-Wnt1 mice) known to be enriched in BCSCs (Tsukamoto, Grosschedl et al. 1988, Li, Welm et al. 2003, Liu, McDermott et al. 2004, Honeth, Bendahl et al. 2005).
Preliminary analysis of different tumor subtypes using IHC with an affinity purified LBH antibody confirmed that LBH protein is preferentially overexpressed in tumor cells of basal subtype, TNBC (Fig. 5.6). In contrast, in HER2+ tumors LBH is only sometimes expressed in tumor associated fibroblasts and is mostly absent in Luminal ER+/PR+ tumors specimen (Fig. 5.6). Expression of LBH specifically in tumor cells of basal subtype TNBCs, highlights LBH as a potential biomarker marker for this type of cancer (Fig 5.6) and together with our functional and molecular analysis implicates LBH as an advantageous novel therapeutic target.

Previous studies have suggested that LBH expression is correlated with a BCSC phenotype (CD44$^\text{hi}$CD24$^\text{lo}$) and that RNAi KD of LBH in TNBC lines leads to a more differentiated (CD44$^\text{hi}$CD24$^\text{hi}$) antigenic phenotype (Rieger 2010), however, the effects of LBH on BCSC activity were not clear. The functional analysis described here showed that the tumorsphere forming capacity of TNBC cell lines was markedly reduced upon RNAi KD of LBH in vitro (MDA-MB-231; HCC1187; MDA-MB-157; Fig 5.8A). Also striking was the increase in tumorsphere formation upon overexpression of LBH in MCF-LBH and BT549-LBH (Fig. 5.8B), indicating that LBH plays an important role in both promoting and maintenance of BCSCs. Similar to our findings in normal MaSCs, molecular analysis of ΔNp63 revealed the same positive upstream relationship whereby a decrease in LBH resulted in decreased ΔNp63 (Fig. 5.9A) and conversely an increase in LBH resulted in an upregulation of ΔNp63 (Fig. 5.9B). Such a regulatory mechanism in which LBH functions to upregulate ΔNp63 has
important implications for the self-renewal of BCSCs as ΔNp63 is already known to be an important stem cell maintenance factor in normal MaSC (Li, Singh et al. 2008, Yalcin-Ozuysal, Fiche et al. 2010). Furthermore ΔNp63 has been shown to positively regulate the Wnt/β-catenin pathway and nuclear accumulation of β-catenin through inhibition of GSK3β-mediated phosphorylation and degradation of β-catenin (Patturajan, Nomoto et al. 2002), as well as the TGFβ pathway through direct positive transcriptional regulation of TGFβ1 ligand and other superfamily components (Lindsay, McDade et al. 2011, Balboni, Hutchinson et al. 2013). This may suggest that LBH maintains stem cells by inducing a positive feedback circuit with these stem cell pathways via ΔNp63.

Identification of such regulatory pathways in BCSCs is of grave importance as these cells are typically resistant to traditional therapies (Jones, Matsui et al. 2004, Creighton, Chang et al. 2010) and subtypes of breast cancer enriched in BCSCs such as basal-like, cannot be treated with traditional endocrine therapies due to a lack of expression of previously identified targetable receptors (ER/PR/HER2) (Honeth, Bendahl et al. 2008). Remarkably, in addition to reduced functionality of BCSCs upon depletion of LBH (Fig. 5.8A), we also observed a striking increase in expression of luminal differentiation and positive prognostic marker ERα (Fig. 5.9C), indicative of a transition to a more differentiated luminal phenotype in the absence of LBH. The converse relationship was also true: ERα decreases when LBH is overexpressed in breast cancer cell line MCF7 (Fig. 5.9D) and suggests that the previously observed inverse correlation between LBH overexpression and ERα in primary breast
tumors (Rieger, Sims et al. 2010) may be due to active repression of ERα by LBH and ΔNp63. The mechanism by which LBH represses ERα is still unclear but may be mediated through the combined effects of regulation on multiple levels. One possibility is that LBH may be affecting protein stability by increasing proteasomal degradation and thus resulting in decreased ERα protein half-life. Additionally, as our qPCR data would suggest, LBH may be playing a role in regulating transcription of ERα through modulating the milieu of transcriptional regulators present at the ERα promoter and moreover could potentially result in changes to the promoter methylation status as is commonly observed in ER-human breast tumors (Ottaviano et al. 1994, Lapidus et al. 1998). Furthermore, mRNA stability could be affected through LBH-mediated regulation of microRNA’s that act upon the 3’ UTR of ERα, however, future studies will need to be performed to address these potential modes of LBH-dependent ERα regulation. One exception to these findings were our results in MDA-MB-157 cells, which despite efficient KD of LBH (Fig. 5.7A) displayed no change in ΔNp63 expression (Fig. 5.8A). Interestingly, no changes in ERα were observed in MDA-MB-157 either, supporting the notion that the observed regulation of ERα by LBH is through a ΔNp63-dependent mechanism as our experiments in non-transformed MEC would suggest (Fig. 4.12).

Basal breast cancers have also been shown to possess an EMT-like signature and increased invasiveness and metastatic potential (Sheridan, Kishimoto et al. 2006, Shipitsin, Campbell et al. 2007, Fillmore and Kuperwasser 2008). Investigation into the migratory and invasive properties of breast cancer
cell lines upon depletion or overexpression of LBH revealed that LBH is also pro-invasive (Fig. 5.10). Together these studies highlight LBH as a potential novel biomarker for basal-like, TNBC with an important functional role in the maintenance of an undifferentiated, invasive, BCSC phenotype. Future studies will need to further address the ability of LBH to affect BCSC genesis and maintenance in vivo using a genetic approach by crossing Lbh knockout mice we have generated (Lindley and Briegel 2013) with MMTV-WNT1 mice, which develop undifferentiated tumors enriched in BCSCs (Li, Welm et al. 2003, Liu, McDermott et al. 2004, Watanabe, Fallahi et al. 2013) and express high levels of Lbh (Rieger, Sims et al. 2010).
Chapter 6: Summary and Future Directions
6.1 Summary

LBH is a developmentally expressed, tissue-specific transcription cofactor in vertebrates that acts in the WNT signaling pathway, a genetic program critical for embryogenesis and adult tissue homeostasis. Aberrant gain-of-function of LBH is implicated in both human congenital disease and breast cancer. Mounting evidence suggests important roles for LBH as a transcriptional co-regulator during embryonic development, mammary gland (MG) tissue homeostasis/stem cell maintenance and cancer (Briegel and Joyner 2001, Briegel, Baldwin et al. 2005, Conen, Nishimori et al. 2009, Rieger, Sims et al. 2010). However, the precise function of LBH in development and breast cancer was not known. Using a novel conditional LBH knockout mouse model and tissue culture models of MG development, we have identified that LBH is implicated in MaSC expansion/maintenance and the repression of luminal-specific genes. Additionally, we have identified that LBH promotes its stem cell effects, at least in part through induction of epithelial stem cell transcription factor ΔNp63. Further work is needed to investigate the exact mechanism by which LBH regulates or interacts with ΔNp63. Moreover, since LBH is aberrantly overexpressed in cancers with WNT hyperactivation (basal breast, colon-unpublished), it will be important to determine whether LBH contributes to the oncogenic stem cell effects of Wnt/b-catenin signaling in vivo.

The normal physiological function of LBH had been elusive due to a lack of genetic loss-of-function models. To study the in vivo roles of LBH, we have generated mice with a conditional null allele of Lbh by flanking exon 2 with loxP
sites \((Lbhf_{\text{fox}})\) in Chapter 3 (Lindley and Briegel 2013). Homozygous \(Lbh_{\text{fox}}\), and \(Lbh_{\text{foxP}}\) mice, in which the Neo cassette was removed through FLPe-mediated recombination, were viable and fertile, indicating that these conditional \(Lbh\) alleles are fully functional. \(Lbh_{\text{foxP}}\) mice were then crossed with a \(Rosa26-Cre\) line resulting in ubiquitous deletion of exon 2 and abolishment of LBH protein expression. Mice homozygous for the \(Lbh\) null allele \((Lbh^{\Delta 2})\) displayed normal embryonic development and postnatal growth with morphologies indistinguishable from wild-type littermates. However, MG development, which occurs primarily after birth, was significantly delayed (Lindley and Briegel 2013). Prompted by these findings we further investigated a role for LBH during mammopoiesis in Chapter 4 with specific focus on mammary stem cells (MaSC), as these are the drivers of pubertal MG development (Watson and Khaled 2008).

To specifically gain insight of LBH in breast epithelial development and oncogenesis, we generated an epithelial-specific \(Lbh\) knockout mouse model by crossing \(Lbh_{\text{foxP}}\) mice with a \(Keratin14-Cre\) (\(K14-Cre\)) deleter strain, which expresses Cre predominantly in basal epithelial cells. In Chapter 4 we showed that in wild-type (WT) mice, LBH is predominantly expressed in the MaSC-enriched \(CD29^{hi}CD24^{lo}\) basal cell subpopulation of the MG. \(K14-Cre\) mediated \(Lbh\) knockout results in a pronounced delay in pubertal MG outgrowth, similar to ubiquitous \(Lbh\) deficiency. Furthermore, luminal differentiation was abnormally increased at the expense of basal myoepithelial differentiation. These defects could be traced to a severe reduction in the number and self-renewal/differentiation potential of MaSCs. Mechanistically, we identified LBH as
a positive upstream regulator of epithelial stem cell transcription factor ∆Np63, promoting a basal stem cell phenotype and repressing expression of luminal cell-specific genes. Taken together, these studies establish LBH as an essential regulator of normal MaSC maintenance and basal lineage specification, thus raising important questions for its potential role in the pathogenesis of breast cancer and breast cancer stem cells (BCSCs).

In Chapter 5, we follow up on this question by establishing an important role for LBH in promoting an invasive BCSC phenotype. First we showed that LBH is rapidly induced during TGFβ mediated epithelial to mesenchymal transition (EMT) of human mammary epithelial cells (HMEC), suggesting that LBH is involved in EMT mediated dedifferentiation of MECs and is also a direct target of the TGFβ pathway. In vitro studies using breast cancer cell lines (MDA-MB-231; HCC1187; MDA-MB-157) demonstrated that LBH expression is required for the maintenance of a BCSC phenotype, whereas overexpression of LBH promotes stemness in MCF7 and BT549 tumor cell lines. We also show that LBH is pro-migratory and pro-invasive in breast cancer cells, as knockdown (KD) of LBH in MDA-MB-231 and MDA-MB-157 breast cancer cells reduced migration and invasion, while overexpression in MCF7 and BT549 increased migration and invasion. Lastly, molecular analysis confirmed the same upstream relationship between LBH and ∆Np63 in repression of ERα in breast cancer cells as was observed in normal MaSC. Together our findings highlight LBH as a potential therapeutic target for anti-cancer stem cell therapies that upon inhibition is predicted to induce the differentiation of BCSCs.
6.2 Future Directions

Despite the advances in understanding the physiological role of LBH described in this thesis, many questions remain about the molecular mechanisms underlying LBH function, especially in vivo. Therefore in the remainder of this chapter I will outline experiments that might be interesting to perform in the future.

6.2.1 Etiology of reduced litter size displayed by ubiquitous Lbh null females

In addition to the observed striking delay in pubertal MG development, a marked reduction of the alveolar compartment in pregnant, lactating and involuting $Lbh^{Δ2/Δ2}$ females was observed as compared to stage-matched WT glands, suggesting that lobulo-alveolar development was perturbed in these mice (Lindley and Briegel 2013). Moreover, pups from $Lbh^{Δ2/Δ2}$ mutant females were significantly less viable independent of their genotypes than pups from $Lbh^{+/+}$ and $Lbh^{+/Δ2}$ females, which may indicate insufficient milk provision by these mothers due to attenuated differentiation of lobuli into secretory alveoli. Typically, $Lbh^{Δ2/Δ2}$ mutant females had an average litter size of 4.6±0.5 pups at the time of weaning (3 weeks of age), whereas $Lbh^{+/Δ2}$ control females had 7.6±0.7 pups (n=12 litters per group; Student’s t-test; p=0.001537). IHC and WB analysis of pregnant and lactating MGs and protein samples from $Lbh^{Δ2/Δ2}$ mutant females using a β-casein specific anti-body as well as qPCR of β-casein transcript levels should be
used to assess if milk production is reduced. Additionally, FACS analysis using antibodies against CD24, CD29/49f, and CD61 should be performed during pregnancy stages to assess if there is an aberrant distribution of MEC populations in Lbh deficient glands. Pregnancy is another stage in MG development, during which MaSCs rapidly expand (Asselin-Labat, Sutherland et al. 2007), hence, a decrease in parity-induced stem cell activation or an increase in terminally differentiated luminal cells could explain the impairment in the functional secretory epithelium and inability to properly nurse pups in Lbh null mice.

6.2.2 Genetic Lineage Tracing Studies to Label LBH Positive Mammary Epithelial Cells In Vivo

Using the combined techniques of WB, IHC, FACS, qPCR and RNA in situ hybridization we have shown LBH expression to be enriched in the basal/MaSC enriched epithelial and stromal populations of the MG, while it is mostly excluded from the luminal compartment (Rieger, Sims et al. 2010, Lindley and Briegel 2013). However, there is currently no experimental method available that allows for direct isolation of pure MaSCs from primary tissues and despite optimizing our IHC protocol for LBH some background staining remains, making it impossible to completely appreciate the expression pattern of LBH in the MG. One strategy that could provide useful in elegantly delineating the expression pattern of LBH and for use in downstream techniques would be to employ a genetic GFP “knock-in” approach similar to that used for Lgr5-GFP-CreER\textsuperscript{T2}
reporter mice (Barker, van Es et al. 2007). Using this approach, GFP would be expressed from the *Lbh* promoter resulting in GFP positive cells where *Lbh* is normally expressed. This would allow for visualization of LBH expression in situ and would also permit direct isolation of high purity LBH+ cells using FACS analysis, which could further be used to characterize their stem cell activity by in vivo transplantation assays.

6.2.3 *In Vivo* Mammary Transplantation Assays with *Lbh* Deficient or Enriched Mammary Epithelial Cells

Thus far we have used *in vitro* methods to test the effects of *Lbh* deficiency on MaSC function: serial mammosphere and differentiation assays. Although these methods unequivocally demonstrated that LBH is necessary for proper function of MaSCs and their self-renewal, the ‘gold standard’ technique for assessment of MaSC defects is through isolation of MECs and transplantation into cleared fat pads of recipient mice *in vivo* as was first described by DeOme et al. more than 50 years ago. Ideally for our purposes, MaSC enriched CD29hiCD24+ MECs isolated from WT and *Lbh* null MGs using FACS analysis would be injected into cleared fat pads of recipient mice followed by serial passaging of these cells in host mice. This would allow us to evaluate the *in vivo* effects of *Lbh* depletion on the repopulating capacity of MaSC. Alternatively, to avoid any sort of bias in cell population distribution during development and to assess the immediate effects of *Lbh* deficiency on MaSC repopulation capacity, primary MaSC enriched CD29hiCD24+ MECs from WT mice could be isolated
using FACS followed by infection with lentiviral or adenoviral particles with *Lbh*-specific shRNA to ablate *Lbh* expression just prior to injection into recipient cleared fat pads. Conversely, this technique could be used to ectopically introduce LBH into luminal cells to validate that LBH positivity enriches for basal cells with stem cell activity *in vivo*, as has been done for the basal transcription factor (TF) Slug (Guo, Keckesova et al. 2012).

6.2.4 Targets of LBH Regulation and Interaction

LBH has transcriptional co-regulator activity both in vitro and in vivo (Briegel and Joyner 2001, Briegel, Baldwin et al. 2005, Ai, Wang et al. 2008, Conen, Nishimori et al. 2009). However, due to lack of a DNA binding domain and only limited knowledge of interacting partners, identification of direct transcriptional targets has remained a challenge. Therefore, a candidate approach based on observed phenotypes has led us to the identification of ΔNp63 and ERα as potential targets of transcriptional regulation by LBH

**ΔNp63 and ERα**

The *in vivo* studies based on our *Lbh* null mice provide the first compelling evidence that LBH acts upstream of ΔNp63, as *Lbh* deficient MECs have a striking reduction in ΔNp63 transcript and protein. A positive relationship between LBH expression and ΔNp63 was also observed in both normal MECs and breast cancer cell lines at the mRNA and protein level. The p63 locus has two isoforms that are expressed from differential promoter usage: ΔNp63 and TAp63. Hence,
it is plausible that LBH acts as a switch to regulate p63 promoter usage by repressing the Tap63 and activating the deltap63 promoter through differential binding with co-repressors or activators. LBH possesses a high degree of structural disorder and conformational plasticity, which allows LBH to interact with numerous binding partners (Al-Ali, Rieger et al. 2010). A first approximation of whether LBH is affecting expression at the promoter level would be possible through the use of luciferase promoter constructs with both the TAp63 and ΔNp63 promoters and measurement of changes in luciferase signal in the presence or absence of LBH. This could be accomplished using HC11-LBH and vector control cells (HC11-vector) or by co-transfection of HC11 with the respective p63 promoter constructs and a pCDNA-LBH expression vector. To directly assess if LBH is bound to the promoter regions of p63, ChIP analysis could be performed with primer sequences that scan different portions of the p63 promoter sequence using normal human and murine MEC cell lines or breast cancer cell lines that express endogenous LBH (MCF10A, 226L, MDA-MB-231, HCC1187) or ectopically overexpress LBH (HC11-LBH, MCF7-LBH). Interestingly, TCF/lef binding sites have been identified within the p63 promoter regions (Osada, Park et al. 2006) raising the possibility that LBH, as a WNT-induced TF, might cooperate with TCF/beta-catenin in p63 promoter regulation (Rieger, Sims et al. 2010).

ERα is overexpressed in >70% of breast cancers (BC) and serves as an important prognostic marker and target for anti-hormone therapies. Our observation that LBH represses ERα transcription in numerous in vivo and in
vitro normal MEC and breast cancer systems, confirms previous studies by Megan Rieger, who showed that ER protein is downregulated in HC11-LBH cells and LBH overexpression in BC is inversely correlated with ER expression in human breast tumors and tumor cell lines (Rieger 2010, Rieger, Sims et al. 2010). Together these data suggest that aberrant overexpression of LBH in breast cancer may play a pivotal role in tumorigenesis of bad prognosis ER-breast tumors as well as promoting progression of good prognosis ER+ tumors into more malignant ER- tumors. Our KD studies in HC11 might suggest that LBH and ΔN63 may act synergistically possibly by direct protein-protein interaction to repress ERα gene expression (Figure 4.12). For example, KD of p63 in HC11-LBH cells, which have a ~50% reduction in ERα mRNA and almost non-existent protein, resulted in restoration of ERα levels to that of vector controls. However, KD of ΔNp63 in HC11-vector control cells (which lack endogenous expression of Lbh) had no effect on ERα protein or transcript levels, suggesting LBH expression is required for this result. To investigate the possibility that LBH and ΔNp63 interact at the protein level, co-immunoprecipitation (Co-IP) using affinity purified LBH anti-body or ΔNp63 antibody could be employed to pull out putative complexes of LBH and ΔNp63 followed by WB analysis to confirm interaction. Additionally, transient co-expression studies evaluating the effects of LBH and ΔNp63 on the ERα promoter activity should be performed to determine, whether LBH and ΔNp63 directly interact to affect ERα expression.
Identification of New LBH Targets and Interacting Partners- A Global Approach

Previous studies and this thesis work have identified numerous potential targets regulated by LBH including ANF in heart development (Briegel, Baldwin et al. 2005) Runx2 and Vegf in bone development (Conen, Nishimori et al. 2009) and ERα and CD24 in the breast (Rieger, Sims et al. 2010) (Rieger 2010). However, it is not known whether LBH directly controls transcription of these genes and what its other target genes might be. Therefore, identification of LBH target genes in a less biased and more global way would allow for unforeseen targets and also allow for appreciation of LBH global regulation on gene networks and pathways. To achieve this, KD of LBH using RNAi would be performed in normal MEC and/or breast cancer cell lines in which endogenous LBH is expressed at high levels followed by isolation of total RNA and micro-array analysis to identify transcripts that are up or down-regulated upon LBH modulation. These targets could then be validated by qPCR. Alternatively, ChIP-seq analysis (Valouev, Johnson et al. 2008, Visel, Blow et al. 2009), could be used to identify locations of LBH binding throughout the entire queried genome. In this assay, LBH would be immunoprecipitated with affinity purified LBH antibody followed by next generation sequencing of precipitated DNA. In both experimental approaches it would be advantageous to use multiple cell lines and/or primary cells and combination experiments using WNT treatment could help to identify genes downstream of LBH and WNT.
Lastly, identification of interacting protein partners would also further our understanding of the physiological and pathological roles of LBH. Of note, one previous study has indicated that LBH and αβ-crystallin, a small heat shock protein and molecular chaperone, interact and functionally repress p21 and p53 (Deng, Li et al. 2010). However, it would be advantageous to perform co-IP of endogenous or ectopically overexpressed LBH from cell lines or primary cells followed by resolution on an SDS-page gel and mass-spectrometry to identify additional partners in the future.

6.2.5 In Vivo Functional Role for LBH in Breast Cancer

Together our functional data strongly suggest that LBH promotes a BCSC phenotype by increasing ‘stemness’ and invasive capacity. Future studies should focus on evaluating if LBH mediates this effect in vivo using both a genetic approach, xenograft models and tail-vein injections to assess an effect on metastatic potential.

Lbh as a Downstream Effector of WNT-Mediated Dedifferentiation and Oncogenesis

Our previous work showed that LBH is overexpressed in WNT driven tumors from MMTV-Wnt1 mice (Rieger, Sims et al. 2010) which accumulate stem and progenitor like cells overtime (Li, Welm et al. 2003, Liu, McDermott et al. 2004, Watanabe, Fallahi et al. 2013). Recent reports describing the effects of genetic crosses into MMTV-Wnt1 mice to abrogate expression of Pygo2, another
WNT driven factor, failed to prevent accumulation of stem and progenitor like cells in these mice (Watanabe, Fallahi et al. 2013). Given the role we have delineated for LBH in normal and neoplastic MaSC function *in vitro*, the effects of \( Lbh \) depletion in this system should be of extreme interest. This could be accomplished by crossing our conditional \( K14-Cre Lbh \) null mice into the \( MMTV-Wnt1 \) background followed by evaluation of tumor incidence, metastasis and BCSC content. To avoid any artifacts in these parameters due to the effect of \( Lbh \) depletion on MG development prior to tumor formation, \( Lbh^{loxP} \) mice could be crossed into a line containing a Cre recombinase that is active only in the presence of exogenously provided stimuli such as Tamoxifen responsive or antibiotic activated forms of Cre recombinase. This would allow for time-controlled depletion of \( Lbh \) in this system. These data would shed light on the potential role of Lbh in mediated the oncogenic effects of WNT.

**Xenograft and Tail Vein Injection Studies**

We have shown that modulation of LBH in breast cancer cell lines results in increased ‘stemness’ in tumorsphere assays and increased invasion and metastasis using transwell assays. However, these studies to date have relied on *in vitro* measures of the effects of LBH and future studies will need to employ the same cell lines and their response *in vivo*. Stable KD cell lines, preferably inducible, should be used for xenograft transplantation into immune-compromised mice. Tumor formation as well as differentiation status (ER\( \alpha \); Keratin5/8; FACS analysis) should then be assessed to determine if LBH was
sufficient to affect tumorigenicity and differentiation of tumor cells \textit{in vivo} as this could have important prognostic implications. Lastly, using stable or inducible LBH-KD cell lines tail vein injections should be employed to assess the effects of LBH depletion on the ability of cancer cells to colonize the lungs and form micro-metastasis.

6.3 Final Conclusions

Collectively, our studies identified the first physiological role of LBH in stem cell regulation and basal lineage specification in the adult mammary gland. It will be of interest to investigate whether LBH plays a more general role in adult stem cell regulation, as WNT signaling does. Moreover, our studies in breast cancer systems further support an important role of LBH in the formation and maintenance of hormone-receptor negative BCSCs. This knowledge could be exploited to develop LBH into a molecular target in CSC therapy to prevent and treat currently incurable forms of cancer. Based on our collective findings we have derived the following working model outlined in Figure 6.1.
Figure 6.1: Model for LBH regulation and function in normal and neoplastic stem cells. LBH expression is induced by canonical WNT and TGFβ signaling and functions to mediate the stem cell effects of these pathways through induction of ΔNp63. Depletion of LBH and subsequently ΔNp63 in normal mammary stem cells (MaSC) leads to differentiation down a luminal biased lineage. Hyperactivation of WNT and/or TGFβ in cancer leads to overexpression of LBH and an increase in breast cancer stem cells (BCSCs). Self-renewal of BCSCs is propagated in a feed-forward loop whereby LBH mediated ΔNp63 induction leads to positive regulation of WNT and TGFβ signaling. Depletion of LBH in BCSC leads to re-expression of therapeutically “targetable” marker ERα, and may serve as an attractive means of differentiation therapy in BCSCs.
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